Final Report: Subcontract No. XAW-4-14292-01

DEVELOPMENT OF ALTERNATE PRETREATMENTAND BIOMASS FRACTIONATION PROCESSES

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Final Report
Subcontract XAW-4-14292-01

to:

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Summary

Pretreatments of a whole-tree hybrid poplar chipped feedstock and of switchgrass feedstock were performed in series of steady-state reactions using dilute nitric acid and temperatures from 160 °C to 170 °C.

In Section 1 the Experimental Plan is noted with reference to the Annual Report presented in 1995.

NREL's Chemical Analysis and Testing Standard Procedures and modification of these approved by NREL are discussed in Section 2.

In Section 3 pretreatment of switchgrass including steps taken in procurement, preparation and storage and chemical characterization are discussed. Maintenance and modification of the Continuous Flow Reactor Unit (CFHU); i.e., the system including all ancillary equipment and apparatus, are discussed. In particular, attention is given to the prehydrolysis (Stage 1 or S-1) Continuous Flow Hydrolysis Reactor (CFHR) of this system. Designation of the steady-state reaction interval is presented under Section 3, Pretreatment. Dilute nitric acid pretreatment including CFHU operation and CFHR reaction conditions are discussed. The problems of feeding both feedstocks but especially switchgrass and the effect of the high-pressure steam boiler limitation on progress made are discussed. The careful attention given to storage of pretreatment products is outlined.

SSF was carried, Section 4, out using the pretreated solid residues of two hybrid poplar steady-state reactions, two switchgrass steady-state reactions, unextracted switchgrass feedstock and alpha-cellulose. Results have been discussed in some detail. The residues were found to be good substrates for enzymatic hydrolysis in all but one reaction. A satisfactory analysis of the unusual result obtained in this case in spite of an extensive examination of data collected has not been found.

Pretreatment Liquor Fermentation using *Pichia stipitis*, Section 4 was carried out to determine dry cell mass, net ethanol production and products produced. Series of fermentations were carried out in which 0%, 40%, 80% and 90% of the synthetic mixture of sugars used was replaced with the sterile filtered hydrolysate from two steady-state hybrid poplar reactions and two steady-state switchgrass reactions. No inhibition of fermentation was found at 40% hydrolysate concentration; whereas, extensive inhibition was found at 80% and 90%. Acclimation of the yeast to the hydrolysates or detoxification of the hydrolysates are possible solutions to inhibition.

Preface

This study was directed to the hydrolysis of hybrid poplar and switchgrass feedstocks using dilute nitric acid in the pretreatment or first stage to remove hemicelluloses. Adaptation of the continuous flow hydrolysis system to each feedstock and preparation of prehydrolysis residues under steady-state reaction conditions have been carried out. Cellulose present in the pretreated residue or acid insoluble residue from the first stage was converted to ethanol by simultaneous saccharification and fermentation (SSF) in the cellulose (second stage) hydrolysis. Saccharification was carried out using Spezyme CP and fermentation was carried out using *Saccharomyces cerevisiae NREL D_5A*. Fermentation of the monosaccharides in the prehydrolysis liquor was carried out using *Pichia stipitis NRRL Y11545.*

This study is an extension of work that has been carried out over the last two decades at the University of California Forest Products Laboratory on the production of ethanol from biomass.

In the previous work the continuous-flow hydrolysis unit (CFHU) served as a nominal half ton per 24-hour demonstration unit. Numerous feedstocks were evaluated. One of the objectives of this work was to evaluate the technical feasibility of producing ethanol by SSF using the pretreatment residue from hybrid poplar and switchgrass. A second objective was to demonstrate the fermentation to ethanol of monosaccharides in the pretreatment liquors.

The support of NREL in providing financial support in this work is gratefully acknowledged and appreciated. Also the technical consultations with D. Hsu and T. Ehrman and the administrative expertise and guidance of M. Yancey, D. Koepping and E. Oster have been enjoyable and professional and have greatly facilitated this work.

The participation of UCFPL personnel W. Li, X. Liang, C. Luo, R. Singh, and W.Xu, who were graduate students in the Wood Science and Technology Group of the University of California Graduate Division, has been essential in this work.

The financial technical and administrative support provided by members of HFTA including G. Craig, W. Higbie, S. Lynn are gratefully acknowledged and appreciated and have been vital in work carried out.

Finally, I wish to acknowledge the professionalism, wisdom, patience and unflagging energy and dedication that M. Merriman has provided to this project and to the overall program at UCFPL.

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Experimental Plan

The work performed on this project was generally followed as written in the Experimental Plan. The work described in the Statement of Work, in Subcontract No. XAW-4-14292-01, submitted 05/19/94 and accepted 05/23/94 was followed with variances specifically provided for in Appendix A. Both the Experimental Plan and the Statement of Work are included in the final report by reference.

The evaluation of feedstocks in this subcontract was limited by NREL during the initial period of the subcontract to one, switchgrass; the second, corn stover was eliminated.

2. Quality Assurance - Quality Control: QA/QC

A substantial amount of work was directed toward using the applicable procedures detailed in the NREL Chemical Analysis & Testing Standard Procedures Manual; i.e., CATSP Nos. 001-006, and 008-09.

In the first nine months of Subcontract No. XAW-3-11181-01 the QA/QC protocols used at UCFPL were modified as described in the Annual Report (Brink, 1995) submitted 03/04/95. The variances in QA/QC procedures approved by NREL were made to accommodate available facilities at UCFPL and to adapt to analytical procedures that had been developed over a period of years.

Analytical methods falling under Task 2, which had not been, established as CATSP protocols had been added by NREL to their QA/QC procedures. These are included according to NREL nomenclature under Task 2 in this report as Laboratory Analysis and Testing Task - Laboratory Analytical Procedure (LAP). In addition to the LAP protocols NREL had developed a protocol for fermentations with *Pichia stipitis*. In using this protocol the variances allowed for other QA/QC procedures was applicable.

These variances allowed were discussed in the Annual Report under Section 2.1.8, CATSP No. 008, Rev. #4, 6/4/93. Quality control achieved in using *P. stipitis* fermentations in work carried out under this subcontract is discussed under Subtask 2.2.

2.1 Quality Assurance

The written procedures on quality assurance - quality control were submitted May 8, 1993. The steps that were undertaken in meeting NREL specifications in this task were carried out through June, 1994. A chronology of some of the correspondence and activities related to this with variances approved by NREL are presented below.

Letter of 6/30/93 from M. Yancey to D. Brink with interoffice memorandum from M. Himmel to M. Yancey/N. Hinman attached concerning acceptable but not validated procedures used at UCFPL.

Letter of 7/28/93 from D. Brink to M. Yancey in response to the 6/30/93 letter noted with clarification of certain questions, which arose.

QA/QC Report submitted to NREL 09/23/93.

Conferences in Golden, CO, 09/27/93 - 09/29/93, at the time of the Ethanol Project Review Meeting at Lakewood, CO, between T. Ehrman, D. Hsu, M. Merriman and D. Brink concerning the requirement of NREL to use sugar degradation factors, CATSP No. 002, rev. #1, 8/19/92, section 6.2, so that all contractors results would be on the same basis. NREL's need for this requirement and reporting on this basis was clear. Also, reporting on the summative analysis basis as developed at UCFPL (Kaar and Brink, 1991B) was kept open as an alternative method for purposes of closing material balances required in the statement of work. Until mid January, 1994, priority for work that could be carried on by the team at UCFPL had to be placed on putting the CFHU in operation to process hybrid poplar. However, a substantial amount of work also was carried out to modify UCFPL analytical procedures to meet NREL' protocols. When steady-state

CFHR runs were terminated, 01/20/94, major attention was placed on resolving acceptance of QA/QC procedures in order to proceed with other tasks of the subcontract. As outlined in the January, 1994, Monthly Report problems on QA/QC approval by NREL pertained to results reported by UCFPL on correction factors for monosaccharides using CATSP No. 002 and ash contents of hybrid poplar determined using CATSP No. 001.

Meetings with T. Ehrman 02/07-08/94 at UCFPL concerned analytical methods but, especially, deionization of hydrolysates and use of internal standards in CATSP No. 002. In a fax to T. Ehrman from D. Brink, 02/14/94, the method and chromatographic results for removing calcium ion from a neutralized hydrolysate and use of an internal standard for determination of sugars were considered in order to obtain a variance in CATSP No. 002.

D. Brink sent a fax to T. Ehrman, 03/09/94, comparing results obtained in the analysis of: a mixture of standard sugar solutions; a sample of NREL-QA-Wood-008 subjected to CATSP No. 002 hydrolysis followed by deionization of the hydrolysate and use of an internal standard method for determination of sugars; and a sample of NREL-QA-Wood-008 subjected to strict conformance to CATSP No. 002 protocol.

Fax to D. Brink from D. Hsu, 03/11/94, confirming the telephone communication just preceding the fax that the deionization-internal standard method for the analysis of sugars was approved. This followed a similar confirmation by telephone between D. Koepping and D. Brink. Thus; analysis of feedstock and pretreatment residues (stage 1 residues) using this procedure could proceed.

During June, 1994, the technique used in hydrolysis of lignocellulosic samples and analysis of the sugars produced was substantially improved by increasing the number of samples that could be hydrolyzed at one time to 15. This permitted the analysis of five lignocellulosic samples in duplicate and five sugar standards for determination of the sugar degradation factor.

2.1.1 Chemical Analysis and Testing Standard Procedures

The UCFPL method analyzes for volatiles, by retaining them during the autoclaving step in which 4% sulfuric acid is used to hydrolyze the oligomers of cellulose formed in the previous 72% sulfuric acid hydrolysis.

The UCFPL method for sugars determination (by HPLC) used barium hydroxide as the neutralizing agent, rather than calcium carbonate. NREL had UCFPL adopt the use of calcium carbonate to have the UCFPL procedures conform to the other subcontractors and UCFPL use of barium hydroxide was not continued in this program.

2.2 Quality Control

The experimental work designed to demonstrate accuracy of chemical analysis is reported. In **Figure 1**, the dry cell mass calibration curve obtained in fermentations carried out using *Pichia stipitis* NRRL 11545 demonstrates the quality control achieved in following the quality assurance procedures provided by NREL.

In Table 1 quality control achieved by refining and fractionation of the feedstock is illustrated.

In Table 2 quality control obtained in analysis of duplicate samples of -3/8 inch size switchgrass described in Table 1 show that % standard deviation based on average % of the sugar analyzed were significant.
In Table 10 the analytical values and the corrected analytical values given for the sugars present in the pretreatment residue from switchgrass Reaction 3 meet the requirements for quality control. Thus, duplicate analyses were carried out for each sugar, the average and standard deviations are given as percentages and the correction factor was obtained and used as specified under Section 2.1, Quality Assurance.

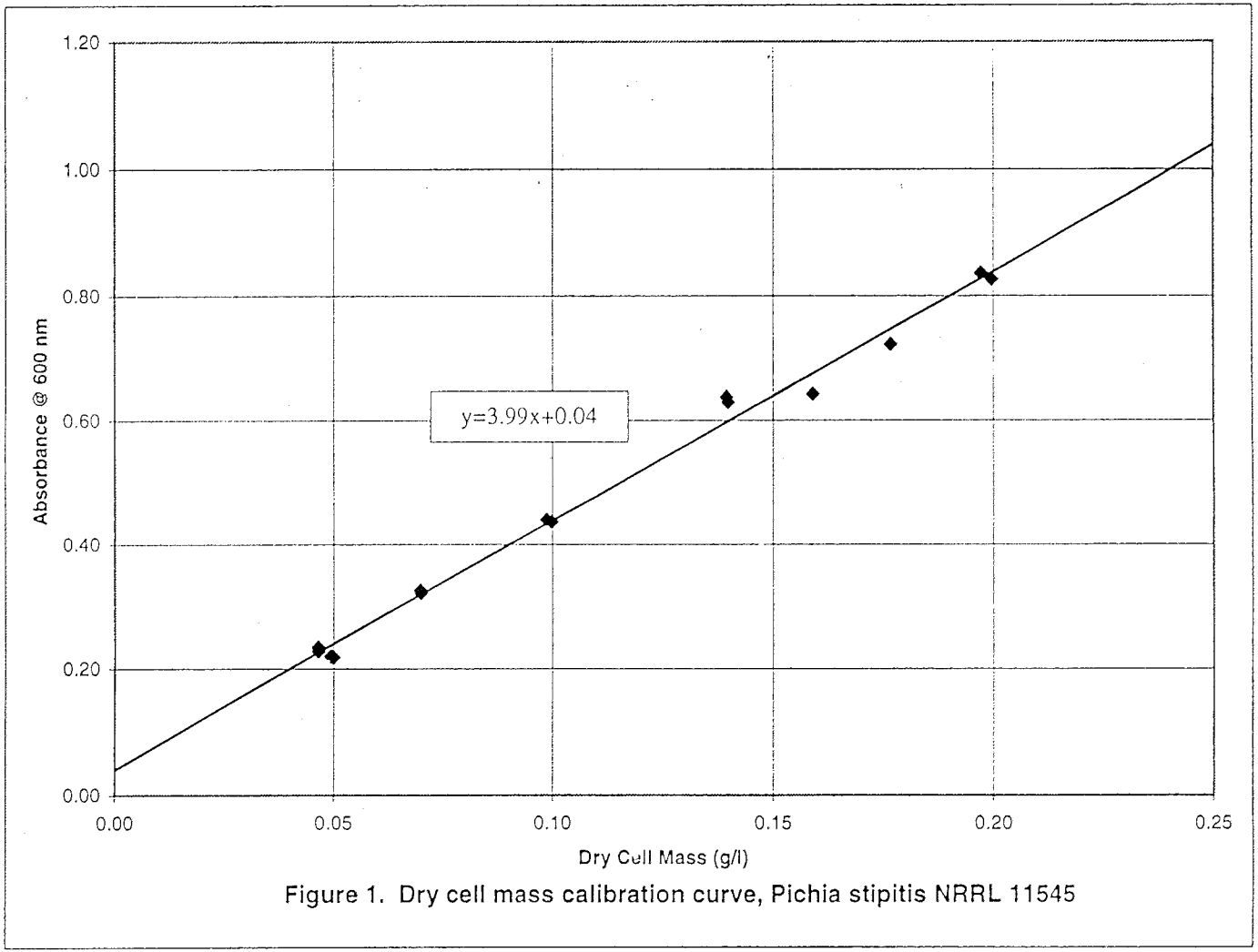

Figure 1. Dry cell mass calibration curve, Pichia stipitis NRRL 11545

3. Pretreatment

Specification of reactions for both study of hybrid poplar and switchgrass has been used to designate the feedstock, the CFHR used, the steady-state reaction number and the interval. Thus HP-1, S1-03, I-1 or SWG-1, S1-03, I-3 are designated in the Annual report as HP Reaction 3 and SWG, Reaction 3. There are seven steady-state hybrid poplar reactions reported in the Annual Report and two steady-state switchgrass reactions reported in the final report. In addition the reactions carried out to establish steady-state intervals in the work on switchgrass are pertinent to the results obtained and are included in the final report. Major emphasis is placed on hybrid poplar Reactions 1 and 3 and on switchgrass Reactions 3 and 10. Either switchgrass or SWG and hybrid poplar or HP may be used in specification of the Reaction. Also, in the hybrid poplar study the S2 CFHR was used; it was not used in the switchgrass study.

3.1 Feedstock

A study was made relative to the size of the switchgrass prepared, the method of size reduction used, and the method of size selection of the fraction used to prepare the feedstock.

3.1.1 Procurement

NREL selected the source of the stock to be used. A sufficient quantity of field dried switchgrass was stored in a covered, dry warehouse with which to supply all of the subcontractors in this study. The samples to be used in the UCFPL program were obtained from this NREL stock on several occasions.

In August, 1994, NREL supplied UCFPL with three polyethylene lined fiber drums of chopped switchgrass from their warehouse stock. Each drum contained from 74 - 85 pounds of classified airdried material in sealed polyethylene bags. One drum contained nominal -3/4 in. material, the second drum nominal -1/4 inch material and the third drum nominal -1/8 inch material.

On 09/20/94 one drum of chopped, -3/8 inch switchgrass was requested from NREL. This feedstock was received on 09/23/94.

On 10/06/94 the milling of the switchgrass feedstock to -1/8 inch followed by screening to remove fines high in ash (-40 mesh) was discussed. A shipment of 1,500 airdried pounds of feedstock processed in this manner was considered. The equipment at UCFPL that might be used for milling the feedstock was considered and discussed with A. Wiselogel of NREL and Len Walde of BePex, Santa Rosa. In further discussions on processing switchgrass feedstock at UCFPL it was concluded that processing on the 18-inch Sweco Vibratory Screen, screening out oversize, hammermilling the oversized followed by screening the material after hammermilling would eliminate the oversized material. Then screening could be carried out to retain a class range for further processing. The fines not retained in the acceptable class range would be discarded. Screening trials were to be run on the 18 inch Sweco Rotary Screen to obtain data on size distribution of fractions as feedstocks were supplied by NREL with no additional hammermilling. The ash content of each fraction obtained was to be determined. This evaluation would establish whether there was a beneficiation of the feedstock in the size reduction classes that would be produced by NREL processing a

portion of the master feedstock supply before shipping to a contractor.

3.1.2 Preparation and Storage

The shipments of switchgrass received by UCFPL, August 1994, were stored in sealed polyethylene plastic bag liners placed in polyethylene lined drums in covered dry storage at UCFPL until used.

3.1.3 Particle Size Distribution

On 09/10/94, VibraScrew Feeder trials were run on the three classes of feedstock samples received from NREL in August. The -1/4 inch and -1/8 inch material were fed reliably without interruption at about 50% of maximum rate achievable using this feeder. The -3/4 mesh material fed unreliably bridging the feeder frequently due to an abundance of stalks of 2 to 4 inches or longer.

Apparent packing density was determined during the 09/10/94 runs by free fall collection of the material from the feeder. The results, expressed as pounds per cubic foot were, 12.59, 9.65 and 6.98 for the -1/8 inch, -1/4 inch and -3/4 inch feedstocks, respectively.

On 09/26/94 , VibraScrew Feeder trials were run on the -3/8 inch feedstock sample received on 09/23/94. Many stems longer than 4 inch caused bridging of the Feeder. By removing the majority of the long pieces and using the maximum clearance in the lockhopper this problem was not overcome.

3.1.4 Chemical Characterization

The chemical composition of the switchgrass feedstock was determined.

On 09/29/94 the effect of wetting the -3/8 inch switchgrass feedstock showed that it swelled by approximately 50% of its initial airdried volume. This will adversely (reduce) effect apparent bulk density in the digester.

A sample of the -3/8 inch switchgrass feedstock of approximately 230 grams was classified using 10, 20, 40, 80, and 170 mesh Sweco screens. A second sample of nominal -1/8 inch switchgrass of approximately 985 grams was classified from the consignment of switchgrass received 02/23/95. The same set of Sweco Screens, just noted was used. Ash content of each fraction was determined to establish whether it changed with mesh size .

Results are given in **Table 1** of duplicate samples of each mesh size. These results show a progressive and significant increase in ash content as the mesh size of the fraction is increased above the plus (+) 40 mesh fractions; i.e., in the -40 mesh and smaller fractions. The -40 mesh material amounted to only about 33% and 8% of the total material of the -1/8 inch and the -3/8 inch feedstocks, respectively. Further, by processing to the smaller mesh size of the feedstock the percentages of smaller fractions were increased. This result provided convincing data that refining the switchgrass provided a feedstock that was not representative of the whole feedstock and would take more energy in its preparation. Countering this result the beneficiated feedstock will consume less acid in the pretreatment stage, and probably provide benefits

relative to accessibility to enzymes, consumption of nutrients in and expense of processing through fermentations, and processing of residues subsequent to fermentation. Further consideration of the effect of this ash on the economics of the process was beyond the scope of this subcontract. These considerations and the information obtained was used by NREL in processing feedstock before shipment and additional processing to be used at UCFPL in the preparation of the feedstock to be used in Stage 1 (prehydrolysis) dilute nitric acid hydrolysis. It also demonstrates the importance and care we placed on the preparation of a grass feedstock. The recognition of problems involved in using a grass feedstock was based on previous experience in processing rice straw and bamboo and a biannual crop, cotton stalks, in the CFHU.

The -1/8 inch switchgrass feedstock was used only in switchgrass Reactions 1 and 2 in which steady-state intervals were not obtained. Based on these trials and with the information on beneficiation available, the -3/8 inch Switchgrass feedstock from NREL was processed at UCFPL to provide a -4 +40 mesh feedstock in all trials, switchgrass Reactions 3 through 10. Steady-state intervals were achieved in reactions 3 and 10.

Commencing June, 1995, duplicate 0.3 g samples of the feedstock, prepared as described in the previous paragraphs, were weighed out to 0.1 mg and hydrolyzed using the Two Stage Sulfuric Acid Hydrolysis for Determination of Carbohydrates, CATSP No. 002, with modifications approved by NREL. This protocol is discussed in Section 2.1.2. Simultaneously, duplicate samples were weighed out for moisture content determination. Duplicate determinations of the monosaccharides present in each of the hydrolysates prepared were carried out by HPLC. The values of the five monosaccharides present in the feedstock determined in each of four HPLC runs and the mean value of these analyses are given in **Table 2.**

In addition, the correction factors determined according to the protocol noted are given in the table with the corrected percentage of each monosaccharide expressed on the basis of the unextracted feedstock. In addition, the correction factors determined according to the protocol noted are given in the table with the corrected percentage of each monosaccharide expressed on the basis of the unextracted feedstock.

Also, the polysaccharidic composition of the extracted, -3/8 inch switchgrass feedstock, expressed as the monosaccharides, is given in Table 2. The feedstock was extracted as specified under Section 2.2 Chemical Analysis and Testing Task - Laboratory Analytical Procedure, 2.2.1, NREL's Standard Method for the Determination of Extractives in Biomass Chemical Analysis and Testing Task - Laboratory Analytical Procedure. LAP-010, 4/22/94, Tina Ehrman.

Characterization of Switchgrass composition was confined to quantitative determination of constituent sugars and components or products of hydrolysis that are involved as metabolites of yeasts. Analyses for additional products of hydrolysis that would permit material balance closures based on summative analyses were not carried out. The reason for this was that additional analytical work to complete a summative analysis was explicitly excluded from the Statement of Work by NREL and only corrected values for carbohydrates should be used.

Table 1. Fraction Analysis of A.D. Switchgrass Feedstock

(Feb. 23, 95 Consignment, nominal 1/8")				
Mesh Size	Air Dry Wt. gm.	% Collected	Ave. M.C. %	Ave. Ash %
(+10)	121	12.3	5.6	3.2
(-10+20)	334	33.9		
(-20+40)	264	26.8		
(-40+60)	174	17.7	7.2	5.3
(-60+170)	68	6.9	7.6	5.8
(-170)	24	2.4	8.1	9.5
TOTAL	985	100		

(Sept. 94 Consignment, nominal 3/8")			
Mesh Size	Air Dry Wt. gm.	% Collected	Ave. Ash %
(+10)	151	65.6	4.8
(-10+20)	29.7	12.9	
(-20+40)	31.9	13.9	
(-40+80)	13.1	5.7	6.7
(-80+170)	2.85	1.24	7.6
(-170)	1.54	0.67	15.7
TOTAL	230.09	100.01	

Table 2. Extracted Switchgrass (-3/8" mesh) Composition Expressed as Monosaccharides

Hydrolysis Sample No.		1	2	Average	Std. Dev.	Corr. Factor
		%	%	%	%	
Glucose	Analysis	29.77	28.83	29.30	0.66	
	Corrected	34.88	33.77	34.33	0.78	1.17
Xylose	Analysis	17.38	17.20	17.29	0.13	
	Corrected	20.95	20.73	20.84	0.16	1.21
Galactose	Analysis	1.38	1.32	1.35	0.04	
	Corrected	1.43	1.37	1.40	0.04	1.04
Arabinose	Analysis	2.14	2.12	2.13	0.01	
	Corrected	2.22	2.19	2.21	0.02	1.04
Mannose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	0.91

(Content as percentage of OD solid)

Data developed on the summative analysis of Hybrid Poplar was almost completed before the directive in the Statement of Work was received. Accordingly, further work to complete the summative analysis on hybrid poplar was not undertaken. However, before terminating the analytical work substantial information had been obtained on the summative analysis of hybrid poplar. The results obtained and reported, 04/06/94, were substantially improved (May - September, 1994). Accordingly, only the results reported in the Annual Report (Brink, 1995) were obtained. The pertinent aspects of this information are included in both Tasks 4 and 5 of the final report.

Corrected percentages of the corresponding monosaccharides for Hybrid Poplar are given in **Table 3**. The corrections applied were obtained according to CATSP No. 002. The summation of the total polyglycans (corrected), the acid insoluble residue ("Klason lignin") and its ash, the acid soluble lignin obtained using an adsorption coefficient of 110, and ash, all determined quantitatively in this analysis, amounted to over 96% of the unextracted feedstock on an oven dried (O.D.) basis.

The quantitative values of acetyl and anhydroglucuronic acids previously determined and reported provided a summation, on a corrected basis, of over 102% as shown in **Table 4** (Table 3 from annual report, Brink et.al., 1993). When Extractives were added the summation was estimated at 105% to 108%. This summation is used as the basis required for closure of the material balance in the final report. Clearly, one or more values reported are erroneously high.

Extractives or extraneous materials are extremely diversified but always present. Determination of extractives was not included in the Statement of Work in Subcontract No. XAW-3-11181-01; accordingly, time was not allocated to determination of this item in the summative analysis. However, to make an estimate reference was made to the extractives content found in hybrid poplar wood and bark by Blankenhorn et al. and summarized in the reference given by Brink et al., 1993. Specifically, the extractives content of wood, bark, and wood/bark (i.e., whole tree material) ranged from 5.0-6.7, 22.7-31.9, and 8.0-10.2, respectively. Thus, a range within which an analytical value for this class of components would be expected to fall, given in Table 4 for purposes of discussion, was most conservative. The summation of all the components for which an analytical value had been obtained was 102.3%, unextracted, O.D. feedstock basis. It was predicted that this value would be from 3 to 6% higher with the addition of the value for extractives. Accordingly, there were values given for constituents in this table which were too high. It was not appropriate to correct the percentages of all components to 100% by deducting an amount from each component proportional to the amount of that component in the analysis given (i.e., justification of results).

It was our opinion that the correction factors applied to each sugar given in this analysis were not appropriate. It is well known that 72% sulfuric acid at 20 to 30°C will initiate dehydration reactions immediately. On the other hand, the oligomers first formed by sulfuric acid at this concentration are not hydrolyzed. To hydrolyze the oligomers the acid is diluted to 4% and hydrolysis is carried out at elevated temperatures for given periods of time depending upon the temperatures used. Thus, the degradation of polysaccharides under the condition of hydrolysis specified in CATSP No. 002 and used in this work is not expected to be as high as the degradation of the monosaccharides subjected to the same conditions. As observed in the correction values the rate of degradation of xylose is higher than that of glucose, i.e., the two correction values of greatest significance in this work.

Table 3. Unextracted Hybrid Poplar Feedstock Expressed as Monosaccharides

Hydrolysis Sample.	1	1	2	2	Average	Std. Dev.	Corr.
HPLC No.	1	2	1	2			Factor
	%	%	%	%	%	%	
Glucose:analysis	43.43	43.41	42.60	42.67	43.03	0.50	
Corrected	47.94	47.91	47.02	47.10	47.49		1.100
Xylose: analysis	13.98	13.06	13.66	12.73	13.36	0.69	
Corrected	17.10	15.97	16.70	15.57	16.34		1.220
Galactose: analysis	1.65	0.94	2.19	1.28	1.51	0.59	
Corrected	1.81	1.03	2.41	1.41	1.67		1.100
Arabinose: analysis	1.71	1.03	2.17	1.59	1.62	0.59	
Corrected	2.13	1.29	2.72	1.99	2.03		1.250
Mannose: analysis	3.20	1.97	2.95	2.86	2.75	0.59	
Corrected	3.54	2.18	3.25	3.16	3.03		1.104

Table 4. Hybrid Poplar Feedstock Composition (corrected)

As Monosaccharides	Mean %[1]	As Polyglycan	%[1]
Hexose		Hexosan	
Glucose	47.49	Glucan	42.74
Mannose	3.03	Mannan	2.73
Galactose	1.67	Galactan	1.50
Subtotal	52.2	Subtotal	47.0
Pentose		Pentosan	
Xylose	16.34	Xylan	14.38
Arabinose	2.03	Arabinan	1.79
Subtotal	18.37	Subtotal	16.17
Total Sugars	70.6	Total Polyglycan	63.2
Lignin			
Acid Insoluble			30.4
Ash			
Acid Soluble			1.59
Ash			1.16
Subtotal			96.32
Acetyl			3.11
Anhydroglucuronic Acids			2.86
Subtotal			102.29
Extractives			3 to 6
Summation			105-108

[1] Based on OD sample milled to -40 mesh (American Standard Screen).

Because all Subcontractors in this program were using CATSP No. 002 using corrected values for reporting monosaccharides the results obtained and given in Tables 3 and 4 are based on corrected values for monosaccharides. Similarly, corrected values of monosaccharides are used throughout this final report.

A study was made to detect the presence of oligomers produced from cellulose in the hydrolysates. No indication of cellobiose or higher oligomers was found in any of the analyses made.

Ash contents of the wood, bark and wood/bark are discussed in Section 3.4.1.1, Composition of the Acid Insoluble Residues.

The values obtained for the pretreated solids from a lignocellulosic feedstock; i.e., either hybrid poplar or switchgrass, were uniform when prepared under the same parameter settings. However, with a change in a major parameter such as usage of nitric acid, substantial variations in pretreated solids composition were obtained. This subject is discussed in Section 3.4.1.1.

3.2 Maintenance and Modification of the CFHR.

The operation of the CFHR is described in detail in the Annual Report (Brink, 1995). It is included in the Final Report by reference.

As specified in the Experimental Plan (Brink, 1994):

The CFHR was to be maintained in operating condition as an ongoing activity throughout the program. This general aspect of maintenance is considered in some detail in section 3.3.1, CFHR Operation. A very limited number of specific items of maintenance were required under section 3.2.1.

3.2.1 Feed Mechanism for Grass

A solid screw was procured for the VibraScrew Feeder to replace the helical screw if could not be made to serve the purpose; i.e.; the common problem experienced was collapse of the screw when oversized material in the feed caused bridging.

A major effort was made throughout this work to adapt the CFHR system to feeding switchgrass. Steps to do this were taken essentially every month.

3.2.2 Modification

The possibility of using ultrasonic absorption principles for detection of solids level in the reactor was investigated to replace the wood level probe system.

Experimental equipment was set up and operated to evaluate ultrasonic detection in the hydrolysis reactor as a means of discriminating between solids and liquid levels. The cost of the equipment to place this system in operation in the S1 reactor was not included in the budget. Also small-scale gamma ray detectors have been made available at reasonable costs. Accordingly, this work was not pursued further.

3.3 Dilute Nitric Acid Pretreatment

The schematic diagram of the pretreatment (i.e. S1) reactor in **Figure 2** shows the configuration that was used in pretreatment of Hybrid Poplar given in the Annual Report as Figure 3 (Brink, 1995).

The overall protocol for operation of the reactor was the same in the pretreatment of switchgrass. Reference is made to the discussion in the Annual Report. The pretreatment reactor was modified extensively before switchgrass reactions were undertaken. Then as work progressed further modifications were made in an effort to provide a system that would process a grass feedstock satisfactorily .

The protocol for operation of the S1 reactor was modified each time the operation of the CFHR was redesigned. The sequencer program was revised for operation of the modified S1 reactor based on the new protocol for operation. Each time the pretreatment reactor (S1R) was to be run, each operator was issued a revised "Continuous Flow Hydrolysis Reactor" protocol. This revised CHFR protocol included all new changes required of each operator for the specific operation being carried out.

3.3.1 CFHR Operation

Maintenance problems and reactor reconfigurations that were undertakén as the work progressed are summarized in this section. These were discussed more fully in the Monthly Technical Status Reports (MTSR) which follow the chronology and rationale for making changes and conclusions relative to the performance of the S1 reactor in treating switchgrass.

Specific items of maintenance included:

Items of the Moyno pump (P-2) were replaced and the pump was maintained as necessary.

Maintenance of the high-pressure acid feed-pump (p-1) involved replacement of its plunger and procurement and replacement and calibration of the over-pressure, limit switch.

The disintegrator was disassembled, inspected, and the blades were weighed to continue the ongoing database concerning their corrosion/erosion. Maintenance on the disintegrator included repacking of the shaft and replacement of the gear involved in determination of rpm.

Final maintenance steps were taken during March 1995 for operation of the S1 reactor system as a unit in hot tests to be followed by reactions feeding switchgrass.

Cold and hot tests were conducted on the S1 reactor system on 4/10/95-4/11/95 and on 4/15/95 (SWG, Reaction-1). Switchgrass was fed on 4/15/95 but the reaction had to be aborted due to plug formation in the reactor. The "steam" condensate from the lockhopper operation contained solid fines from the feedstock indicating some entrainment of feed. This was confirmed by pH measurement of the condensate, 1.92 collected during the run 4/15/95.

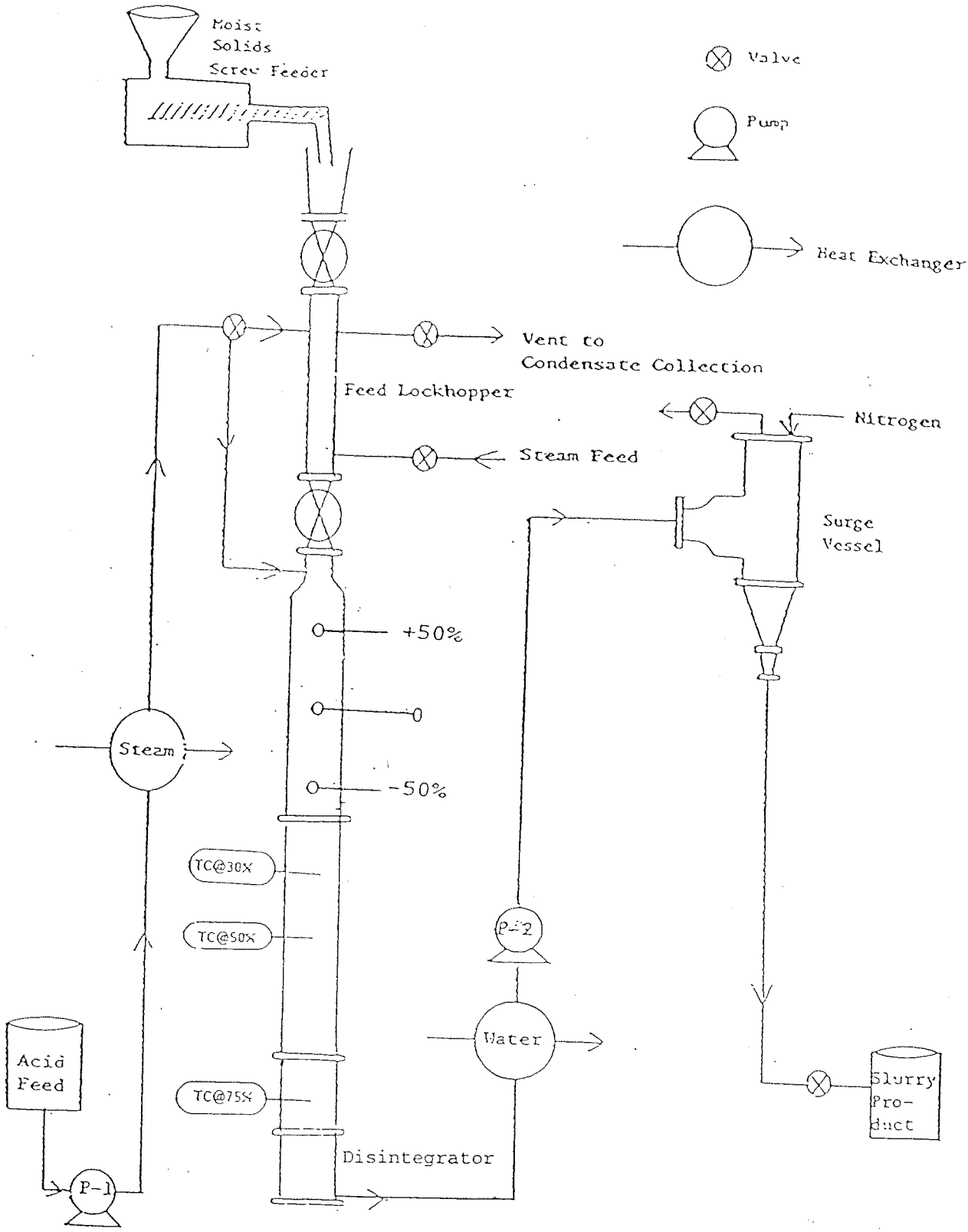

Figure 2. Schematic CFHR used in pretreatment

Steady-state conditions were not achieved in SWG Reaction-1, S-1 due to interruptions in flow through the reactor. In turn there were corresponding interruptions in the rate of feedstock input that resulted in drop in temperature of the slurry in the S1 reactor. This chain of events was attributed to bridges in the feedstock forming between the top of the lockhopper and the funnel through which the feedstock was introduced. Accordingly, extensive maintenance of the S-1 reactor system was continued by undertaking steps to overcome this problem.

An acid reservoir; i.e., a vessel was added which is essentially an extension of the S-1 reactor. This vessel had an internal steam coil used for indirect heating. The vessel was provided with a high pressure Bran and Lubbe displacement pump (P3) to transfer hot acid as a spray into the lockhopper to flush the feedstock into the reactor. The purpose of flushing with acid was to mitigate feedstock plugging in the top of the lockhopper. A long liquid level detector was installed to detect high and low liquid levels. After SWG Reaction-1; a second, short, level indicator was installed to determine high liquid level; the longer probe was used to detect low liquid level. The sensors for the level indicators were calibrated to indicate high and low operating levels and provided with visual monitors and alarms. This provided more positive operational control of the liquid level indication in the acid reservoir. The P3 pump flow rate was calibrated. Thermocouples were installed in the acid reservoir to monitor temperature of dilute nitric acid influent and effluent and of the vapor space.

The 3-way valve was replaced by a two-way valve system for positive control of preheated dilute acid feed from the acid reservoir to only the S1 reactor or to only the lockhopper.

Steam tracing on the S1 reactor and acid reservoir was changed to provide steam at set pressure throughout the repiped system. The pressure transducer in the top section of the S1 reactor was adjusted and recalibrated for detection of liquid level.

The feeding of switchgrass to the CFHR became the major objective to be achieved commencing in June and continuing until September, 1995. Attention was centered on the acid reservoir modification. Previous efforts, which were emphasized had not been successful.

Maintenance of the sequencer and its operation were carried out. during May, 1995, the sequencer settings were changed to include acid reservoir control and acid level indication. In June the system was calibrated to control acid flow.

Cold followed by hot tests were conducted on the S1 reactor system on 06/30/95 (SWG-Reaction 02). No steady-state intervals were run.

Extensive operations of the CFHU were undertaken in July 1995.

Steady-state conditions were achieved 07/03/95 in switchgrass reaction SWG, Reaction 3, I-3. Steady-state conditions were not achieved in SWG 3, I-1 carried out on 07/02/95 or SWG, Reaction 3. I-2 carried out on 07/02-03/95 because of interruptions in flow through the reactor and corresponding interruptions in rate of feedstock input.

In SWG, Reaction 4, 07/12-13/95, the control of the reaction system was not within the limits necessary to be considered at steady state. In another effort to run Switchgrass, SWG, Reaction 5, on 07/16/95 steady-state conditions were not achieved.

Following SWG, Reaction 4 maintenance of the CFHU was continued with an overhaul of the Moyno Pump and recalibration of the surge tank level indicator. More cooling capacity was obtained by doubling the size of the heat exchanger condensing low-pressure steam vented from the lockhopper. This increased capacity decreased the time required to vent and increased the time that acid feed could be sprayed into the lockhopper. All of these actions were carried out to improve feeding Switchgrass to and flow of slurry through the S1 reactor.

No steady-state intervals were achieved in the reactions carried out which included: SWG, Reaction 7, (08/17/95); SWG, Reaction 8, (08/26/95) nor in SWG, Reaction 9 (08/26/95). Problems were incurred in both Reaction 8 and Reaction 9 in maintaining adequate flow through P-3. This led to having to abort the runs in both cases without achieving a steady-state interval.

A major modification in the CFHU S1 reactor was made after SWG, Reaction 9 by eliminating the acid reservoir system. It was concluded that effective and positive flow of acid could be directed to the lockhopper during the high-pressure phase of the sequencer when the bottom 4-inch ball valve was open and to the top section of the S1 reactor during the rest of sequencer cycle. This option was possible due to the positive acid flow that was obtained using the two two-way ball valves in place of the one three-way ball valve, which had not operated satisfactorily.

SWG, Reaction 10, I-1 was carried out on 09/07/95 in which steady-state operation was achieved from a point of view of conditions sustained but which could not be used to establish accurate kinetics. In the reactions leading to SWG, Reaction 10, I-1 maintaining the high-pressure boiler pressure at a satisfactory level had become increasingly difficult. Steps were taken in the boiler operation, which helped maintain pressure at a predetermined level. However, during SWG, Reaction 10 this problem could no longer be solved and the pressure cycled throughout the reaction. None-the-less, a steady-state operation was gleaned from the data being collected during the operation and the start and end of the cycle were obtained. Also, based on the data collected and samples collected, the procedures established for reducing and correlating data have been applicable.

The boiler problem proved to be due to collection of scale in the tube generating the high-pressure steam. The maintenance of the high-pressure boiler had been scheduled but, because of a prolonged down time required for this job, could not be undertaken during the concentrated program to complete reactions from May through September.

3.3.2 CFHR Reaction Conditions

In the acidic hydrolysis of both hybrid poplar and switchgrass feedstocks the objective was to carry out a series of steady-reactions under acidic conditions. In the conduct of work on each feedstock the designations made were for hybrid poplar (HP), the CFHU reactor involved (S-1 or S-2), the steady-state interval. In preparing for SWG, Reaction 1 in April, 1995, titration analyses of switchgrass were carried out

to determine extra acid required to acidify the buffer capacity of switchgrass. An initial nitric acid concentration of 0.30% was selected first.

Steady-state conditions were achieved 07/03/95 in SWG, Reaction 3, I-3.

SWG, Reaction 10 was carried out on 07/09/95. Steady-state reaction parameters are given in **Table 5.**

The major differences in these two reactions were the average temperature, the residence time in the S1 reactor, the nitric acid concentration and the disintegrator speed.

Steady-state parameters are illustrated for Reaction 3. In the figures given, Figures 3 through 11, the feedstock start time (F.S.), slurry collection start time (S.S.), feedstock end time (F.E.), and slurry collection time are given. These times reflect the residence time in the S1 reactor at the nominal reaction temperature. In **Figure 3**, the top temperature at 30% liquid level, **Figure 4**, the central (control) temperature at the 50% liquid level, and **Figure 5**, the bottom temperature at 75% liquid level are given.

Figure 6 provides the reactor vapor pressure. It will be noted that the lower pressure defines the pressure in the vapor space when high-pressure steam is not admitted during the high pressure steaming of the digester; i.e., the 4-inch ball valve is closed and the overpressure of steam has dissipated. The upper points reflect the pressure when the 4-inch ball valve has been opened and live high-pressure steam is being admitted. The lower pressures recorded reflect the change in vapor pressure with temperature in Figure 3; i.e.; this is a dependent variable.

3.3.3 Storage of Pretreatment Products

Two 5 gallon bottles of the acid hydrolysates of each steady-state interval were placed in cold storage at 4°C to be used as required for analytical work or other purposes. The remainder of each acid hydrolysate was stored at the pH of production (about 1.4) in sealed, closed top, 55 gallon, stainless- steel drums in the main building of UCFPL at ambient temperature.

The acid insoluble residues (pretreatment solids) were washed with 0.25% nitric acid to displace hydrolysate and then placed in plastic bags, which were sealed. The plastic bags were placed in cold storage at 4°C.

Table 5. Steady-State Parameters for Switchgrass Reactions

Reaction	Report	Units	3	10
Average Temperature		°C	173.3	167.4
Temperature at Reactor Depths				
30%		°C	173.3	166.7
50%		°C	173.3	167.4
75%		°C	170.3	163.4
Standard Deviations at				
30%		°C	2.0	2.7
50%		°C	1.7	2.6
75%		°C	1.8	1.3
Pressure		psig	145.3	146.85
		SD	22.8	26.23
Nitric Acid Feed	Conc.	%w/w	0.433	1.2
	Rate	kg/min	0.994	1.01
Feedstock Rate	Average	kg/min	0.07	0.07
Residence Time		min	31.59	32.74
Total Steady-State	At Temp.	min	60	90
Disintegrator	Speed	RPM	408.00	691.28
S1 Slurry	Rate	kg/min	1.20	1.16

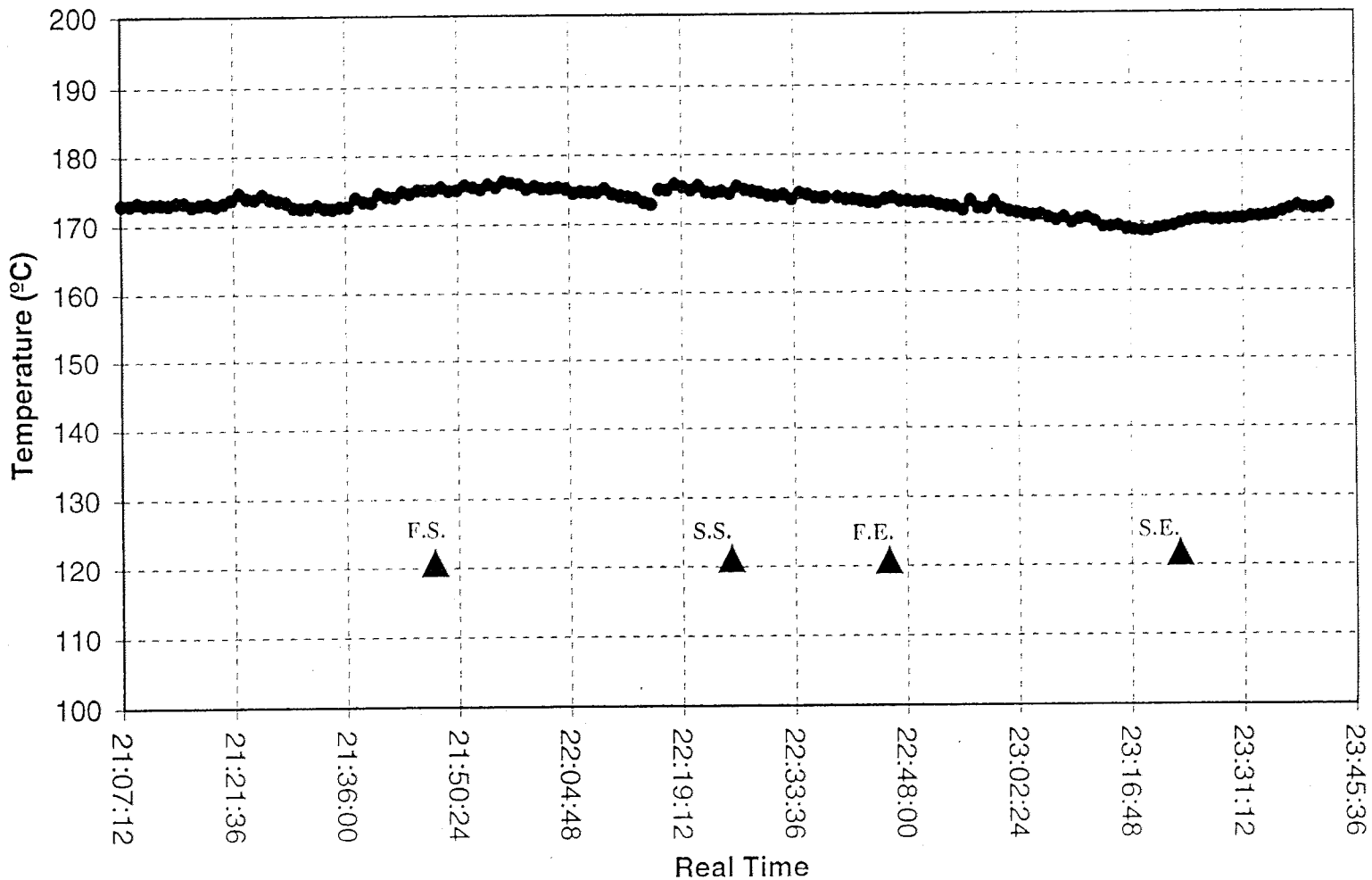

Figure 3. Top temperature at 30% liquid level, switchgrass reaction 3.

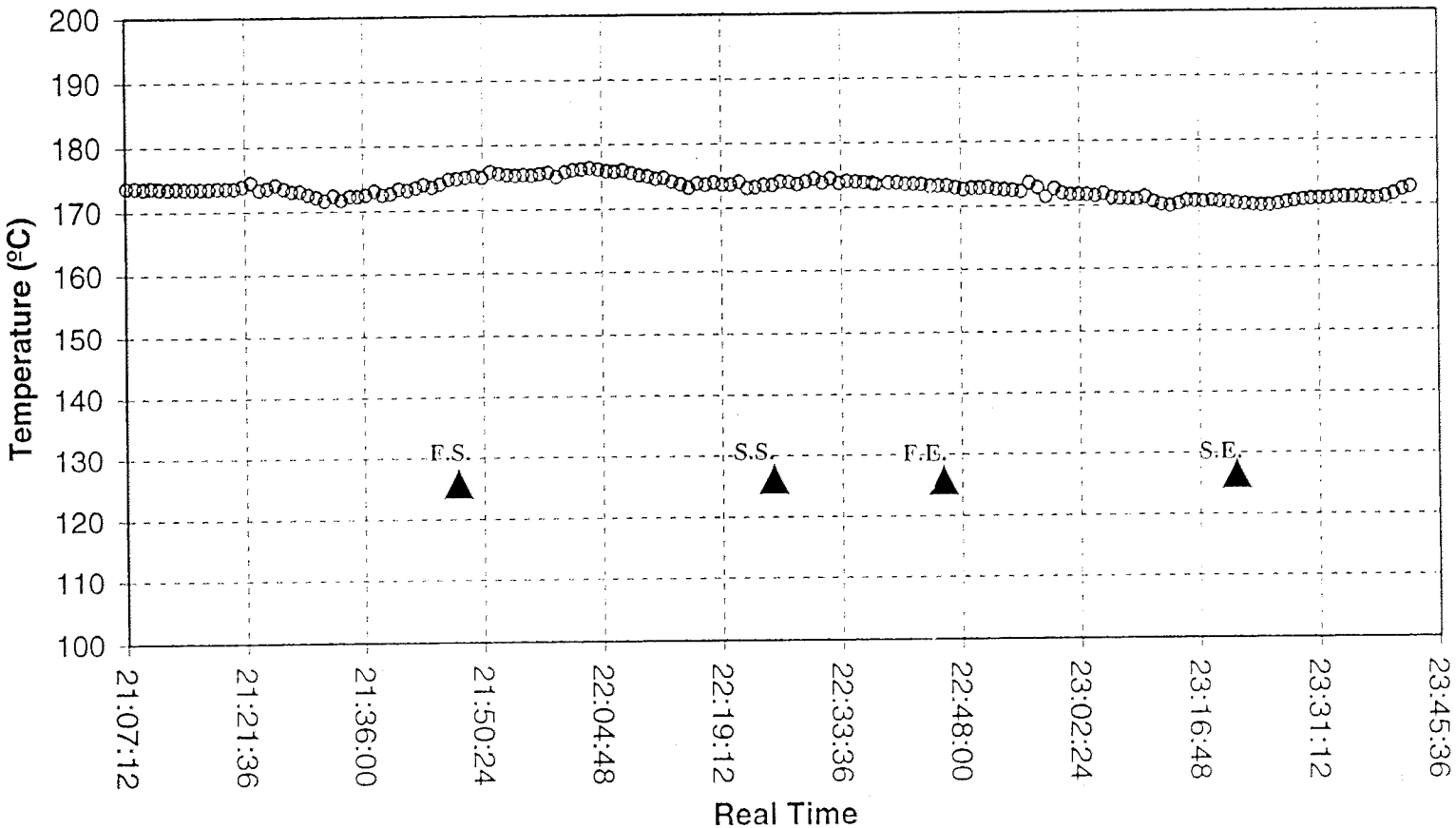

Figure 4. Central (control) temperature at 50% of liquid level, switchgrass reaction 3.

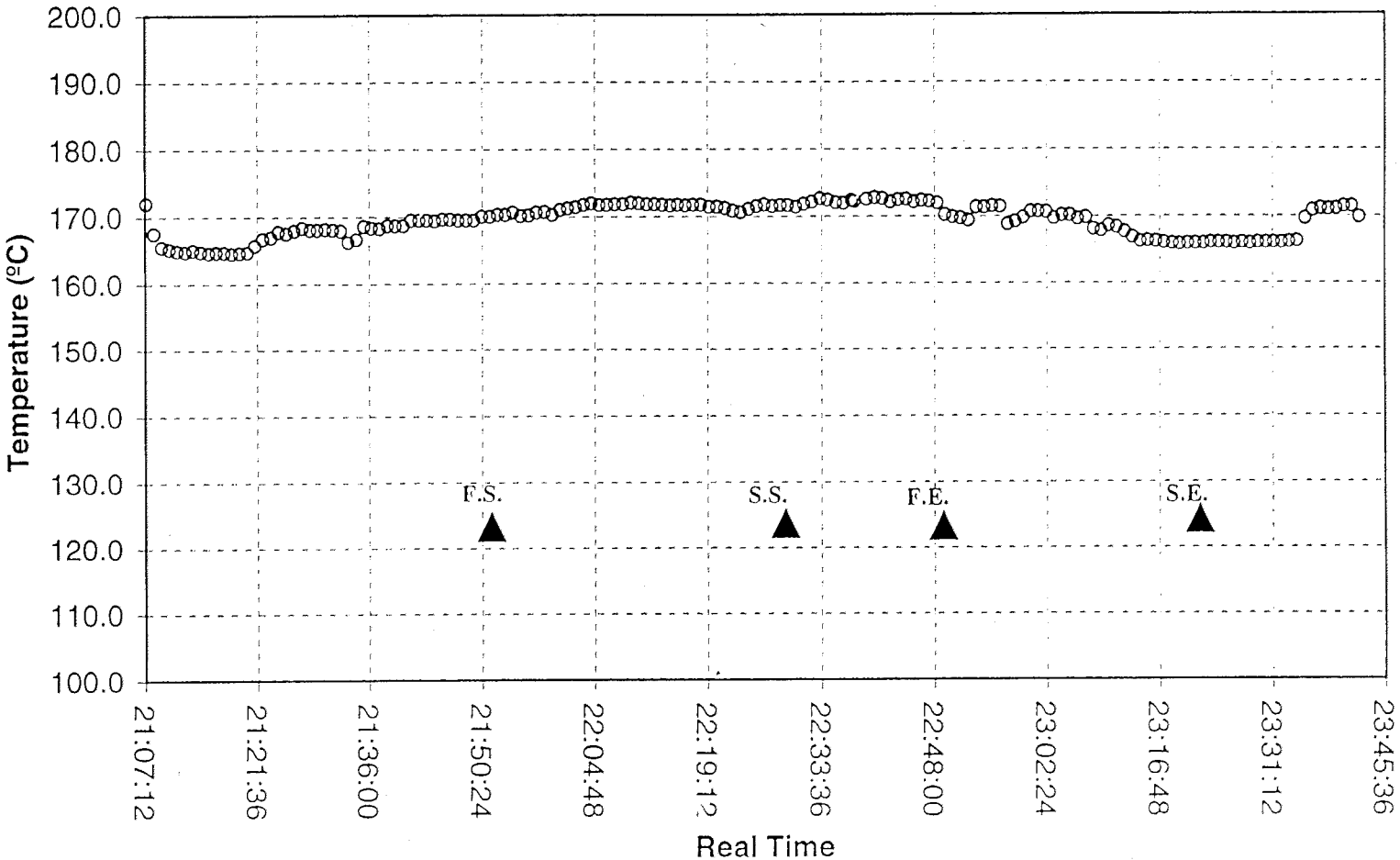

Figure 5. Bottom temperature at 75% of liquid level, switchgrass reaction 3.

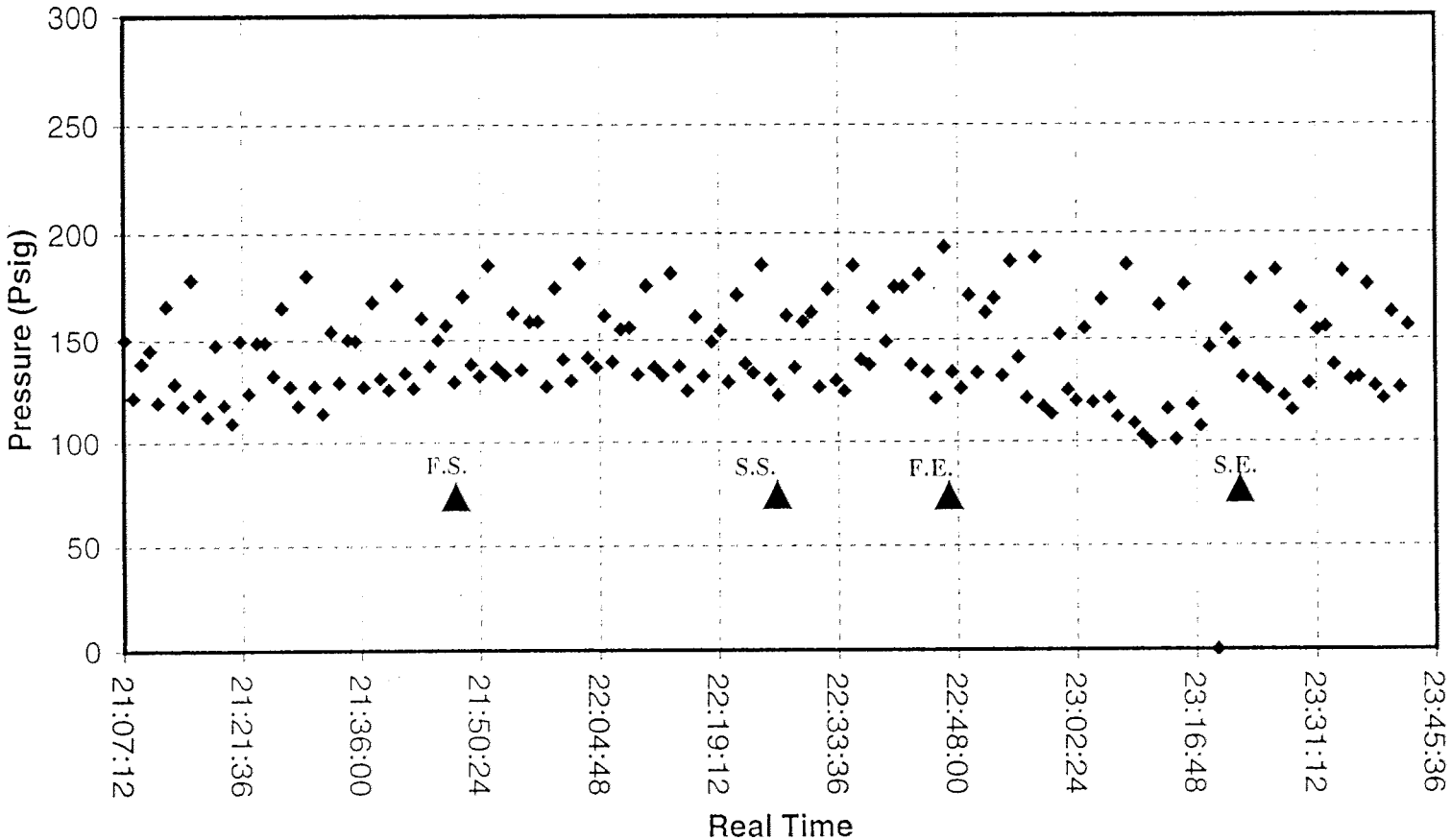

Figure 6. Reactor pressure (vapor phase), switchgrass reaction 3.

3.4 Material Balance Closure

The material balance obtained in SWG, Reaction 3, I-1, given in **Table 6**, 93.4%, showed material balance closure was achieved. However, the recovery of soluble and insoluble solids was low.

The material balance obtained in switchgrass, reaction 10, given in **Table 7**, 17.5%, indicates that very severe conditions were used.

Since the average temperature in this reaction was 6 °C lower than in reaction 3, the substantially higher nitric acid concentration in the feed was the major difference observed. The pH of the slurries was essentially the same. Thus, the "excess" nitric acid did not provide greater conversion but was consumed in oxidation of the components in the concentration of nitric acid present in the reactor in reaction 10.

Cumulative slurry production is illustrated in **Figure 7** for switchgrass reaction 3.

The acid concentration in the slurries produced was calculated from the pH of the slurry assuming an activity of the dilute nitric acid of 1.0.

Feedstock introduced to produce the product slurry was determined as the mass of feedstock added to the reactor in an interval of time initiated before the start of slurry collection. This interval of time, required for a given feedstock sample introduced into the lockhopper to issue as the slurry product in the slurry collection tank, was comprised of two segments. These were the times that the slurry resided at reaction temperature in the reactor and the time for the slurry to pass through the heat exchanger-flow control fume-slurry surge tank and associated tubing system before the slurry discharged to the slurry collection tank. The residence times were unique for each reaction. Accurate correlation of the feedhopper cycle with the slurry discharge cycle and of the liquid levels in the reactor and slurry surge vessel were required to accurately establish the feedstock feed with the slurry produced.

Table 6. Material Balance, Switchgrass Reaction 3

Average Temperature (°C)			173.3
Residence Time in Reactor [at T] (min)			42.6
Steady-State-Interval (min)			63.0
Input	**%**	**Lb**	**Kg**
Feedstock			
Total		10.61	4.81
Rate (mass/min)		0.17	0.08
Moisture Content	8.42		
Water		0.89	0.41
OD Feedstock		9.72	4.41
OD Feed Rate (mass/min)		0.15	0.07
Dilute Nitric Acid			
Total		138.11	62.64
Acid Feeding Rate (mass/min)		2.19	0.99
Concentration of Acid	0.43		
Water (total)		137.51	62.37
Water (mass/min)		2.18	0.99
HNO_3 (total)		0.60	0.27
Rate (mass/min)		0.0095	0.0043
Output:			
Slurry		166.30	75.43
Flow Rate (@ 25°C) (mass/min)		2.64	1.20
Insoluble Solids		1.36	0.62
Hydrolyzate			
Total		159.40	72.30
Soluble Solids	0.63	1.00	0.45
Water		158.40	71.85
Water Gain Lockhopper & Washing		20.00	9.07
Rate (mass/min)		0.32	0.14
Total Soluble + Insoluble Solids		2.36	1.07
Recovery	24.28		

Table 7. Material Balance, Switchgrass Reaction 10

Average Temperature (°C)			167.4
Residence Time in Reactor [at T]			44.1
Steady-State Interval			90.0
Input	**%**	**Lb**	**Kg**
Feedstock			
Total		37.67	17.09
Rate (mass/min)		0.42	0.19
Moisture Content	8.42		
Water		0.04	0.02
OD Feedstock		28.36	12.86
OD Feed Rate (mass/min)		0.32	0.14
Dilute Nitric Acid			
Total		199.47	90.48
Acid Feeding Rate (mass/min)		2.22	1.01
Concentration of Acid:	1.2		
Water (total)		197.08	89.39
Water (mass/min)		2.19	0.99
HNO_3 (total)		2.39	1.09
Rate (mass/min)		0.027	0.012
Output:			
Slurry		229.20	103.96
Flow Rate (@ 25°C) (mass/min)		2.55	1.16
Insoluble Solids		2.43	1.10
Hydrolyzate			
Total		241.40	109.50
Soluble Solids	1.05	2.53	1.15
Water		238.87	108.35
Water Gain in Lockhopper & Washing		41.76	92.07
Rate (mass/min)		0.66	1.46
Total Soluble + Insoluble Solids		4.95	2.25
Recovery	17.47		

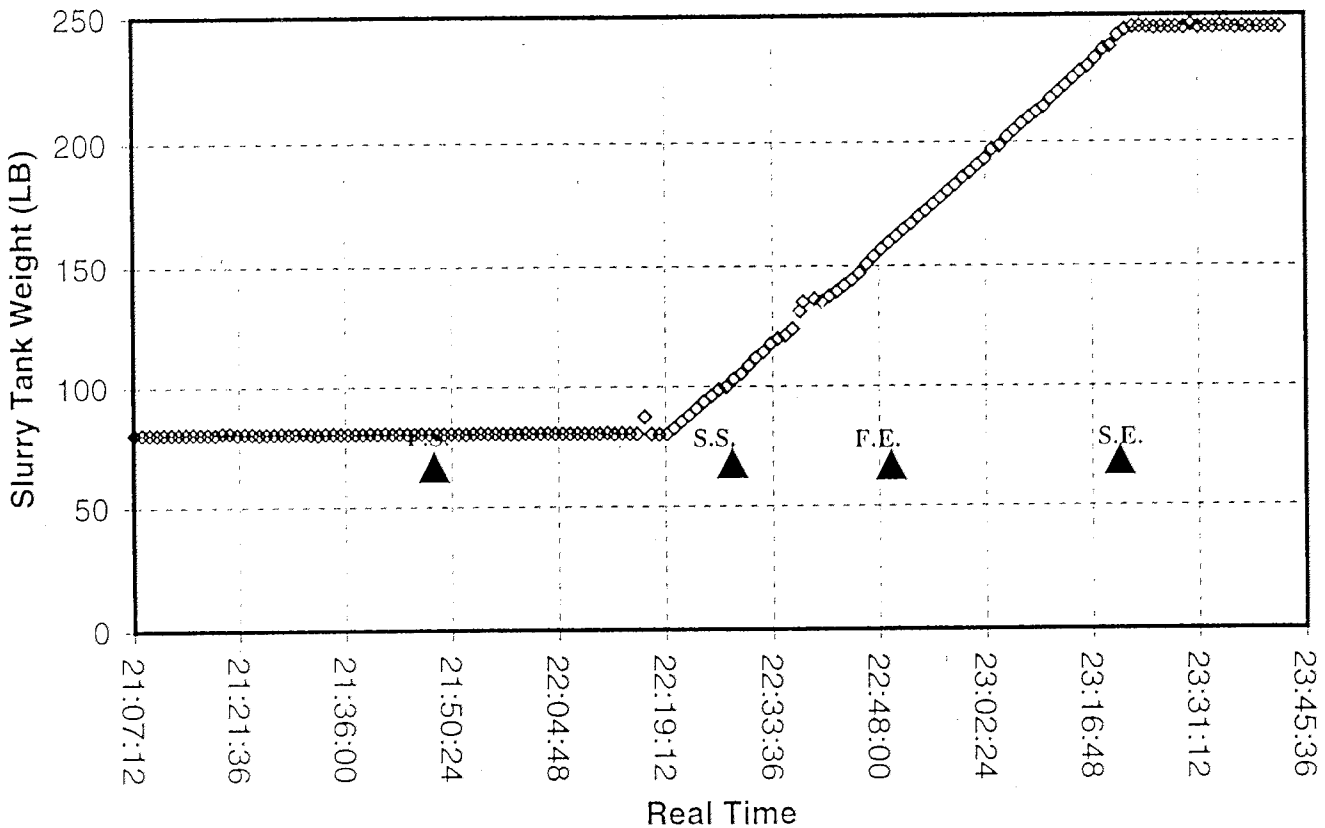

Figure 7. Cumulative slurry production, switchgrass reaction 3.

The cumulative feedstock introduction into the reactor in reaction 3 is illustrated in **Figure 8**.

The slurry collection and VSF weight for switchgrass Reaction 3 are plotted together in **Figure 9.** The slurry collection rate was uniform throughout the steady-state period excepting a slight discontinuity at approximately 22:35 to 22:36 hours showing start and end of feeding to the lockhopper preceded start and end of collection of slurry by the residence time of solids at steady-state temperature in the reactor plus the time from quenching the slurry temperature leaving the disintegrator to collection in a slurry product tank (see Figure 2).

The rate of feedstock introduction over each steady-state interval is calculated from the cumulative feedstock introduced into the reactor and the total time for that interval. These rates for the steady-state reactions carried out are given in Table 5 and are expressed as kg/min. The speed for the disintegrator rotor was 408 rpm for Reaction 3 and 691 for reaction 10.

The rpm at which the disintegrator was operated in Reaction 3 is presented in **Figure 10**.

The power required by the disintegrator is illustrated in **Figure 11** for Reaction 3. It was shown that the power consumption was essentially constant at about 330 watts from F.S. to F.E., the time that slurry is being processed through the disintegrator.

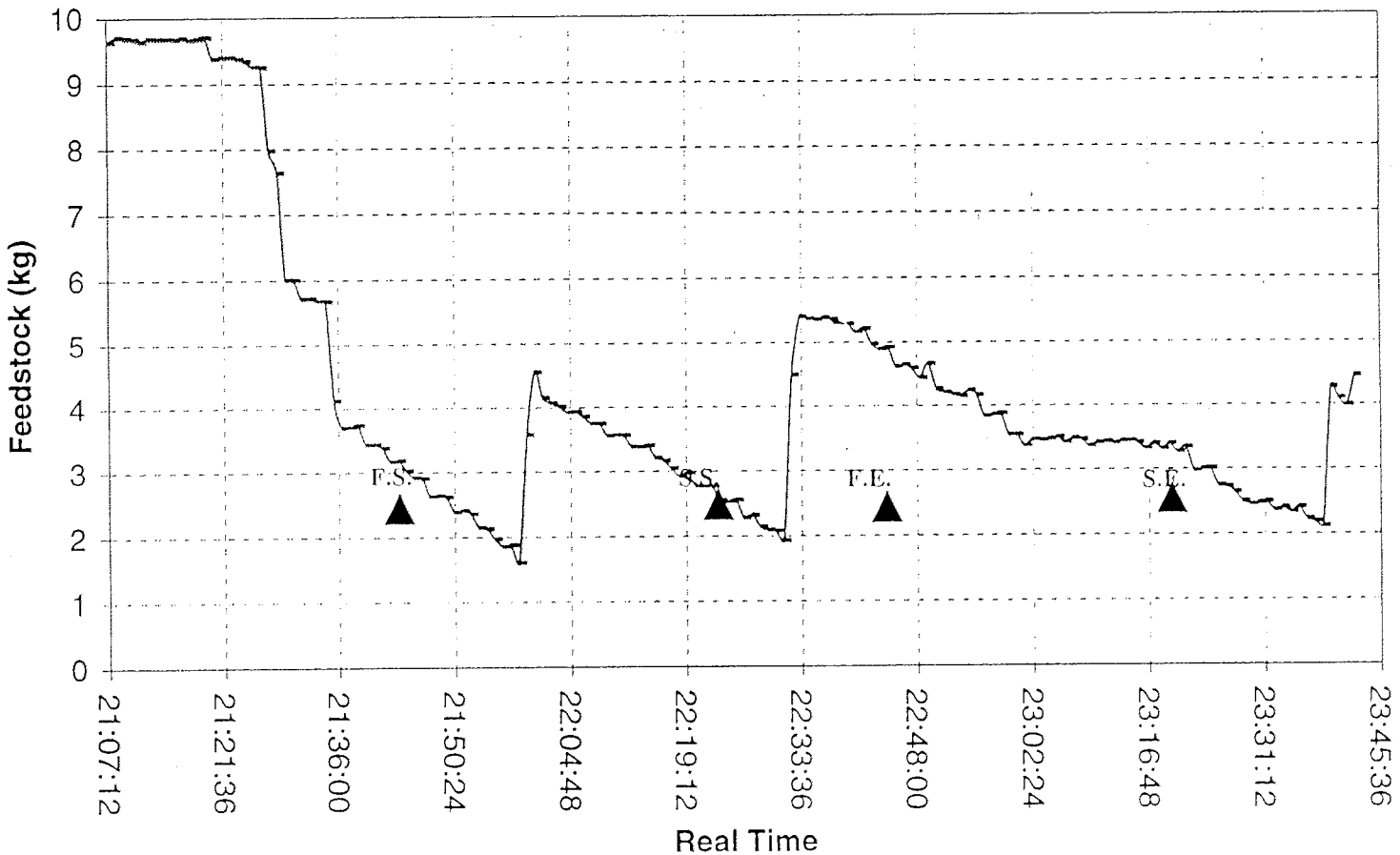

Figure 8. Feedstock introduction, switchgrass reaction 3.

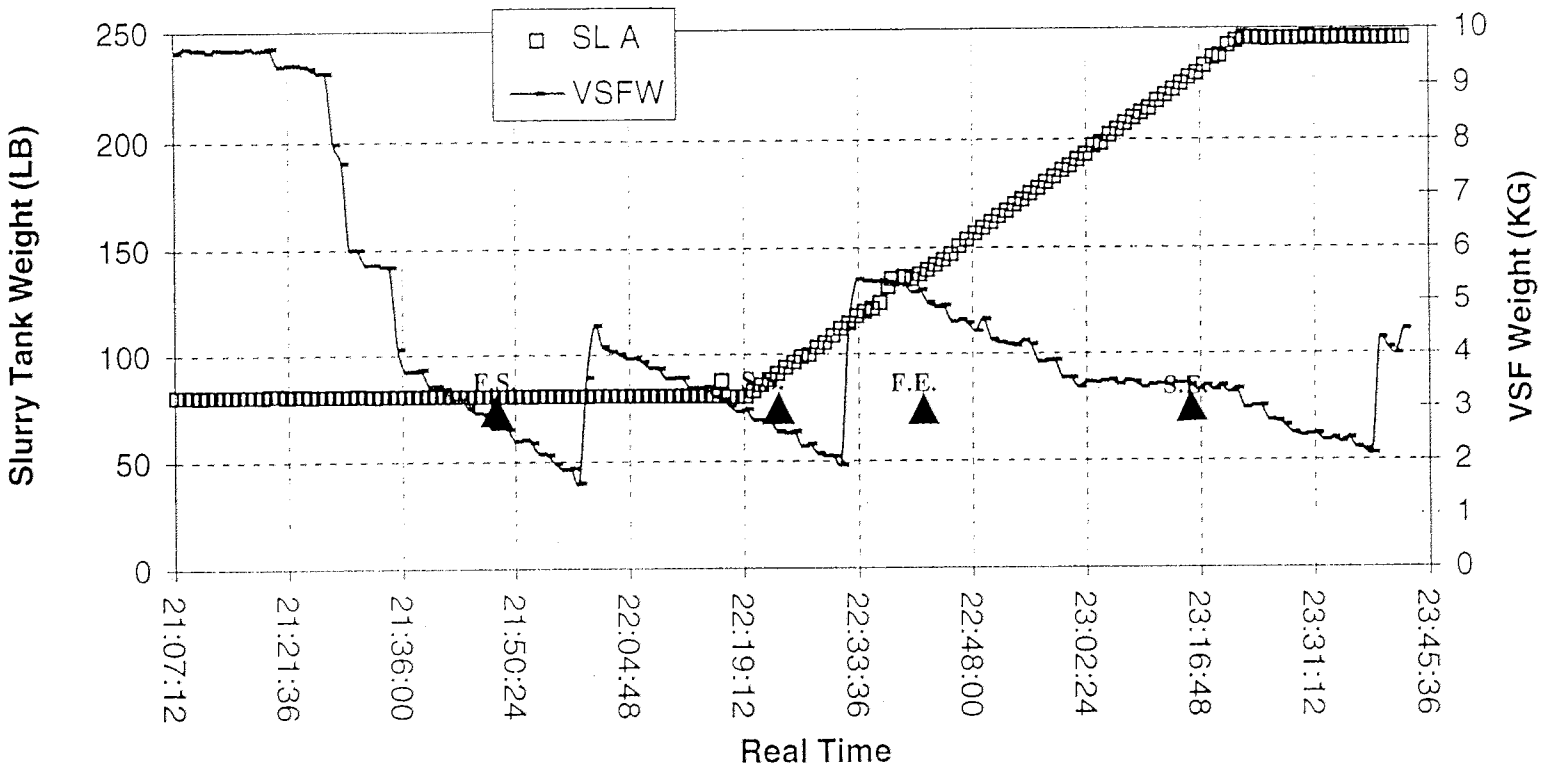

Figure 9. Slurry collection and VSF weight for switchgrass reaction 3.

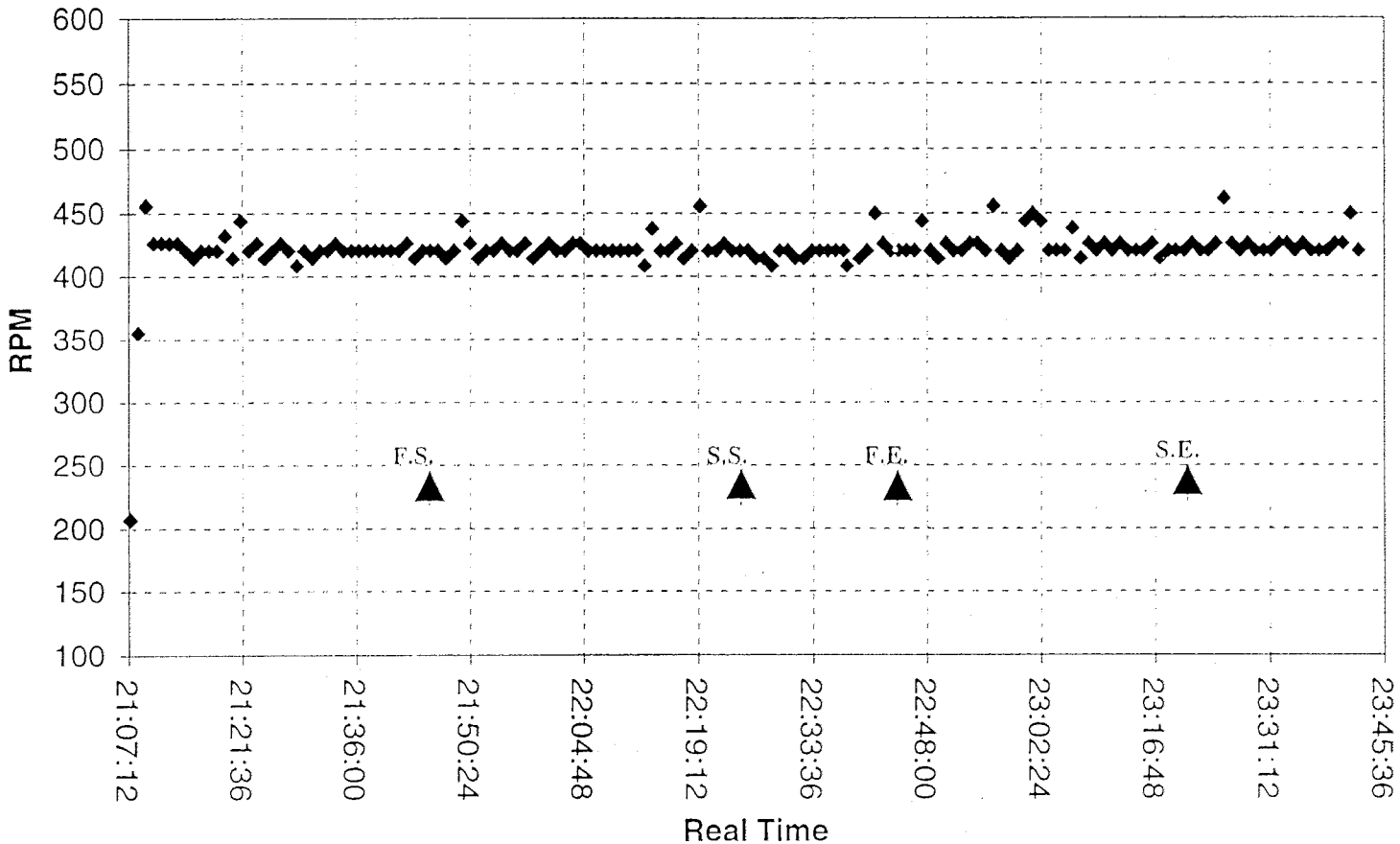

Figure 10. Disintegrator RPM, switchgrass reaction 3.

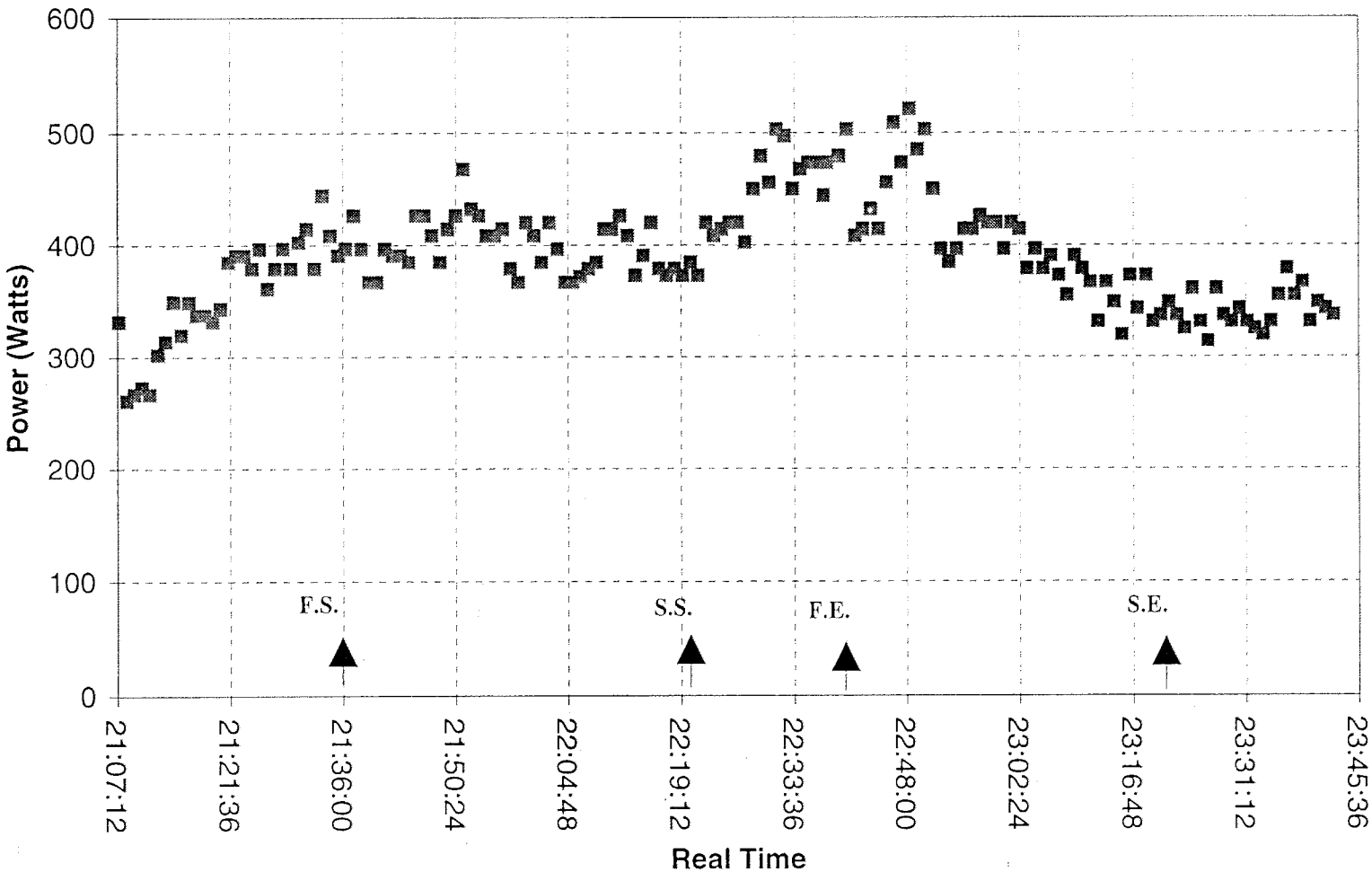

Figure 11. Disintegrator power, switchgrass reaction 3.

3.4.1 Steady-state Intervals

The results obtained in conducting the steady-state reactions described in section 3.3.2 were presented for each reaction as the material balances obtained (Tables 6 and 7), the composition of the acid insoluble or pretreatment residue produced, and the pretreatment liquor composition. Based on these results the hexosan conversion, yield, unconverted and recovery and the pentosan conversion, yield, unconverted and recovery are summarized.

The particle size distribution of the pretreatment residues was obtained for hybrid poplar and was given in the annual report. This property for switchgrass residues was not obtained.

3.4.1.1 Composition of the Acid Insoluble Residues.

The sugars present in the pretreated solids from switchgrass Reactions 3 and 10 are given in **Tables 8 and 9**, respectively. These are reported as both % as monosaccharide and % as polyglycan.

It was noted that in Reaction 3, produced at an average temperature of 173°C, the pretreated solids contained an appreciable amount of xylose. The polyuronide hexoses, mannose and galactose were absent. This demonstrated that the glucomannans, including a small portion of glucose, and pectin, containing galactose, were completely removed.

In Reaction 10, the xylose was also completely removed although the average temperature was only 167°C. This demonstrated the substantially more severe conditions used in Reaction 10 compared to Reaction 3. Since the residence times of the feedstock in the S1 reactor were 32 minutes in Reaction 3 and 32.7 minutes in Reaction 10, the more severe condition was the concentration of nitric acid; 1.2 %w/w in Reaction 10 in contrast to 0.43 %w/w in Reaction 3. Also, the total sugars, as polyglycan amounted to 50.5% in Reaction 3 compared to only 26% in Reaction 10. Thus, the glucose was severely degraded in Reaction 10.

By comparison of results with hybrid poplar, the glucan content varied within the narrow limits of 52.2% to 54.4% with the exception of HP, Reaction 2 at 50.5%, the same as the glucan content of the SWG, Reaction 3 residue. This uniformity of hybrid poplar residues was expected in view of the uniform processing conditions used in the hybrid poplar reactions. The residual xylan content demonstrated the efficacy of hydrolysis of hemicelluloses in reactions 4, 3 and 2, with residual xylan contents of 0.89%, 0.95%, and 0.96%, respectively. Also, the absence of mannose and galactose demonstrated the complete removal of the glucomannans that were present in minor amounts in the hybrid poplar feedstock.

The sugars present in switchgrass acid insoluble residues from SWG, Reactions 3 and 10 are shown in **Tables 10 and 11**, respectively.

The analytical values and the corrected analytical values are given for each sugar. Two analyses were made for each sugar, the average and standard deviation are given as percentages and the correction factors used for each sugar are given.

Table 8. Switchgrass Reaction 3 Pretreated Solid Composition, Monosaccharides and Polyglycans

	As Monosaccharides				As Polyglycan		
	%	Lb	Kg		%	Lb	Kg
Hexose				**Hexosan**			
Glucose	52.71	2.33	1.06	Glucan	47.44	2.10	0.95
Mannose	0.00	0.00	0.00	Mannan	0.00	0.00	0.00
Galactose	0.00	0.00	0.00	Galactan	0.00	0.00	0.00
Subtotal	52.71	2.33	1.06	Subtotal	47.44	2.10	0.95
Pentose				**Pentosan**			
Xylose	3.38	0.15	0.07	Xylan	3.04	0.13	0.06
Arabinose	0.00	0.00	0.00	Arabinan	0.00	0.00	0.00
Subtotal	3.38	0.15	0.07	Subtotal	3.04	0.13	0.06
Total Sugars	56.09	2.48	1.13	**Total Polyglycan**	50.48	2.23	1.01

Table 9. Switchgrass Reaction 10 Pretreated Solid Composition, Monosaccharides and Polyglycans

	As Monosaccharides				As Polyglycan		
	%	Lb	Kg		%	Lb	Kg
Hexose				**Hexosan**			
Glucose	28.85	1.76	0.80	Glucan	25.97	1.58	0.72
Mannose	0	0	0	Mannan	0	0	0
Galactose	0	0	0	Galactan	0	0	0
Subtotal	28.85	1.76	0.80	Subtotal	25.97	1.58	0.72
Pentose				**Pentosan**			
Xylose	0	0	0	Xylan	0	0	0
Arabinose	0	0	0	Arabinan	0	0	0
Subtotal	0	0	0	Subtotal	0	0	0
Total Sugars	28.85	1.76	0.80	**Total Polyglycan**	25.97	1.58	0.72

Table 10. Switchgrass Reaction 3 Pretreated Solids, Duplicate Analytical and Corrected Monosaccharide Composition

Hydrolysis Sample No.		1	2	Average	Std. Dev.	Corr. Factor
		%	%	%	%	
Glucose	Analysis	44.86	45.13	45.00	0.19	
	Corrected	52.56	52.87	52.72	0.22	1.17
Xylose	Analysis	2.86	2.75	2.81	0.08	
	Corrected	3.44	3.32	3.38	0.08	1.21
Galactose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	1.04
Arabinose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	1.04
Mannose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	0.91

(Content as percentage of OD solid)

Table 11. Switchgrass Reaction 10 Pretreated Solids, Duplicate Analytical and Corrected Monosaccharide Composition

Hydrolysis Sample No.		1	2	Average	Std. Dev.	Corr. Factor
		%	%	%	%	
Glucose	Analysis	24.01	25.24	24.63	0.87	
	Corrected	28.13	29.57	28.85	1.02	1.17
Xylose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	1.21
Galactose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	1.04
Arabinose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	1.04
Mannose	Analysis	0.00	0.00	0.00	0.00	
	Corrected	0.00	0.00	0.00	0.00	0.91

(Content as percentage of OD solid)

The ash content of hybrid poplar feedstock, pretreated solids (i.e., acid insoluble residues) and alpha cellulose were given in Table 21 in the Annual Report (Brink, 1995). The ash content of hybrid poplar feedstock was found to be within a normal range for hardwoods. However, the amount of ash present in the pretreated solids was unexpectedly high. This indicated that appreciable amounts of silica or a mineral insoluble under the conditions of pretreatment were present. Furthermore, a high mineral content in the feedstock fines was shown to increase significantly with mesh size; e.g., Table 21 in the annual report (Brink, 1995). Also, the percentages of the fines were shown to vary appreciably from drum to drum of feedstock. This high ash content could have been attributed to the particular composition of the feedstock discussed in Section 3.1.4, Chemical Characterization in the discussion of extractives. Thus, Blankenhorn et. al. (1985) found the bark content of juvenile hybrid poplar to be in the range of about 22% to 32%. The significance is discussed in Section 5.3.

The ash contents of the acid insoluble residues of switchgrass Reactions 3 and 10 were not called for in the experimental plan or for analysis of any product; accordingly, no ashes were determined.

The acid insoluble lignins or more properly, the acid insoluble residues from hybrid poplar were uniform, varying from 38.9% to 41.2%.

This summary of composition of the pretreatment residues produced shows that the process being used produces a product that can be readily duplicated with reasonable controls.

3.4.1.2 Pretreatment Liquor Composition.

The sugars present in the hydrolysates from switchgrass Reactions 3 and 10, expressed as g/l are given in **Table 12.**

Table 12. Switchgrass Hydrolyzate Sugar Compositions for Reaction 3 and Reaction 10

Reaction	Reaction 3	Reaction 10
As Monosaccharides	g/l	g/l
Hexose		
Glucose	1.09	2.57
Mannose	0.34	0.50
Galactose	0.26	0.72
Subtotal	1.69	3.79
Pentose		
Xylose	4.02	7.09
Arabinose	0.54	1.08
Subtotal	4.56	8.17
Total Sugars	6.25	11.96

4. Pretreated Solid Residue Fermentation

This task involves the simultaneous saccharification and fermentation (SSF) of pretreated solid residues using an enzyme to hydrolyze glycans and fermentation of the resulting hexoses by *S. cerevisiae* D_5A to form ethanol.

4.1 Processing and Storage of Pretreated Solids

Reference is made to the preparation of the pretreated hybrid poplar solid residues. The procedure used was described in the annual report in Section 4.2.1, CFHR Operation, with specific statement made that the centrifuged residue was washed with 0.25% nitric acid. Under Task 5, SSF and Enzymatic Hydrolysis Subtask 5.1, Processing and Storage of Pretreated Solids the procedure used is presented. This material was then stored in sealed plastic bags.

The switchgrass solid residues were prepared using the same procedure described in the preceding paragraph. Washing of the centrifuged residues with 0.25% nitric acid is noted under Section 3.3.3 Storage of Pretreatment Products. These washed residues were then placed in plastic bags, which were sealed. The sealed bags were placed in cold storage at 4°C until needed for further work.

4.2 Simultaneous Saccharification and Fermentation

The SSF of hybrid poplar was reported in the annual report under Task 5 (Brink, 1995) and is included in the final report by reference.

Duplicate SSF runs were carried out on each of four substrates; pretreatment residues from switchgrass Reactions 3 and 10; the -3/8 inch, unextracted, -40 mesh switchgrass feedstock; and alpha cellulose (Sigma C8002). The yeast used, *Saccharomyces cerevisiae* D_5A, was cultured from the specimen originally provided by NREL and used in SSF of hybrid poplar.

4.2.1 Enzyme Assay

The cellulase enzyme used was selected by NREL, Spezyme CP. This enzyme was received from Environmental BioTechnologies Inc., Menlo Park, California on 05/12/95, was kept at 4°C and filter sterilized before the enzyme assay.

The enzyme was assayed precisely according to NREL's CATSP No. 006, REV.#1, 08/19/92. The procedure used was discussed in the Annual Report (Brink, 1995) in Section 2.1.6 Measurements of Cellulase Activities.

The activity of the enzyme was determined to be 59 International Filter Paper Units (IFPU). This value was determined 09/29/95. The SSF of the four materials noted in Section 4.2 was carried out from 10/06/95 (0.0 hours) through 10/13/95 (168 hours) with sampling at approximately 24 hour intervals.

4.2.2 Ethanol Production and Yield in SSF

The yields of net ethanol produced, reported as concentrations in g/l, are given for the mean of duplicate samples for each period of analysis used from 0 hours to 168 hours for switchgrass Reactions 3 and 10, for unextracted switchgrass feedstock, and for alpha cellulose (Sigma C8002) in **Table 13**. This table also shows the ethanol yields as a percentage of theoretical.

The data for ethanol production are given in Table 13 and plotted in Figures 12-15. These ethanol yields are based on the analytical results determined by YSI adjusted for zero time ethanol. The actual yields of ethanol and other products are given for each duplicate sample in Tables 14-17. The results given in **Table 14** and **Figure 12** are for switchgrass reaction 3.

Ethanol concentrations in switchgrass reaction 3, that were determined by YSI are shown in Table 14, were decidedly more reproducible than ethanol concentrations determined by HPLC. For this reason the yields obtained by YSI rather than HPLC are discussed.

The maximum concentration of ethanol by YSI obtained at 96 hours was 16.2 g/l. The average concentration of ethanol at 72 hours was 14.9 g/l. The ethanol yields shown in Table 13 are corrected for zero time ethanol. The 0 hour deduction is greater than the average of the two values, 1.97 g/l, given in Table 14. This is the ethanol present in the yeast inoculum and the ethanol which will result from any glucose present in the enzyme, the YP medium, and /or the inoculum. The theoretical yield of ethanol is based upon the hexosan present in the fermentation substrate. Figure 12 shows both the net ethanol yield in g/l and the percentage of theoretical yield at each sampling time during SSF in Reaction 3.

In figure 12 the decrease in theoretical percentage conversion to ethanol following 96 hours probably indicates that ethanol was being lost from the quiescent fermentate during sampling. In Table 13 the average maximum net yield of ethanol produced from the total glycan content, hexosans plus hexoses, in Reaction 3 was 13.73 g/l. This gave a theoretical conversion of 89.5 % in 96 hours. The theoretical conversion was calculated from the experimental results given in Table 14 and Table 10.

It can be concluded based on the average value of theoretical conversion given in Table 13 that the acid insoluble residue produced in switchgrass Reaction 3 was an excellent substrate for SSF. This substrate provided a cellulose surface having a high accessibility to conversion of cellulose to oligomers and glucose.

The yields of ethanol produced in switchgrass Reaction 10 that were determined by YSI are given in **Table 15** and **Figure 13**. In Table 15 it is shown by YSI analysis that ethanol production in the SSF reaction reached a maximum in 96 hours at an average concentration of 20.4 g/l. The average concentration of ethanol at 0 hours was 1.66 g/l. As shown in Table 13 the maximum net ethanol concentration of 18.3 g/l occurred at 96 hours. This gave a 119.4 percentage of theoretical conversion. This excessively high percentage of theoretical conversion is clearly shown in Figure 13.

Table 13. Net ethanol Yield from SSF of Switchgrass and α-cellulose (Sigma C8002)

Switchgrass Reaction 3			Switchgrass Reaction 10		
Time, h	Ethanol, g/l	Conv., %	Time, h	Ethanol, g/l	Conv., %
0	0.00	0.0	0	0.00	0.0
24	8.50	55.5	24	10.05	65.6
49	11.63	75.8	49	16.45	107.3
72	12.43	81.1	72	16.15	105.4
96	13.73	89.5	96	18.30	119.4
121	13.40	87.4	121	16.90	110.3
142	13.09	85.4	142	16.88	110.1
168	12.63	82.4	168	15.80	103.1
Theor. Conv.	15.33	100.0	Theor. Conv.	15.33	100.0

Switchgrass Feedstock Unextracted			α-cellulose (Sigma C8002)		
Time, h	Ethanol, g/l	Conv., %	Time, h	Ethanol, g/l	Conv., %
0	0.00	0.0	0	0.00	0.0
24	3.24	18.3	24	6.65	39.1
49	4.06	22.9	49	10.34	60.7
72	3.98	22.4	72	12.79	75.1
96	4.46	25.2	96	13.56	79.7
121	4.50	25.4	121	15.14	88.9
142	3.97	22.4	142	15.14	88.9
168	3.86	21.8	168	16.69	98.0
Theor. Conv.	17.72	100.0	Theor. Conv.	17.02	100.0

Table 14. Products in SSF of switchgrass Reaction 3

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Acetic Acid HPLC	Lactic Acid HPLC	Glycerol HPLC
0	0	1	2.09	1.99	0.54	1.19	0.00	0.27	0.25	0.24	0.10
		2	2.12	1.96	0.69	1.24	0.66	2.46	0.26	0.26	0.09
24	23.5	1	12.20	11.20	0.40	3.40	2.02	2.50	0.37	0.18	0.34
		2	10.76	10.75	0.40	0.00	0.32	1.80	0.37	0.99	0.17
48	49.5	1	22.26	14.55	0.10	0.43	1.50	2.83	0.68	0.17	0.45
		2	12.04	13.65	0.13	0.34	0.72	1.57	0.38	0.08	0.20
72	71.7	1	22.79	15.10	0.05	0.06	0.72	1.87	0.72	0.17	0.47
		2	16.19	14.70	0.06	0.01	0.51	1.15	0.49	0.08	0.24
96	95.8	1	15.68	16.00	0.03	0.00	0.67	2.03	0.47	0.15	0.30
		2	15.59	16.40	0.03	0.00	0.40	1.73	0.49	0.08	0.27
120	120.6	1	22.43	15.85	0.03	0.00	0.28	2.17	0.73	0.15	0.42
		2	15.51	15.90	0.03	0.00	0.16	1.28	0.47	0.08	0.23
144	142.0	1	18.02	15.29	0.03	0.02	0.81	1.53	0.59	0.08	0.35
		2	19.20	15.84	0.03	0.00	0.63	1.59	0.63	0.15	0.37
168	168.0	1	13.17	15.10	0.04	0.00	0.31	1.11	0.43	0.08	0.19
		2	18.66	15.10	0.04	0.00	0.52	1.50	0.62	0.08	0.33

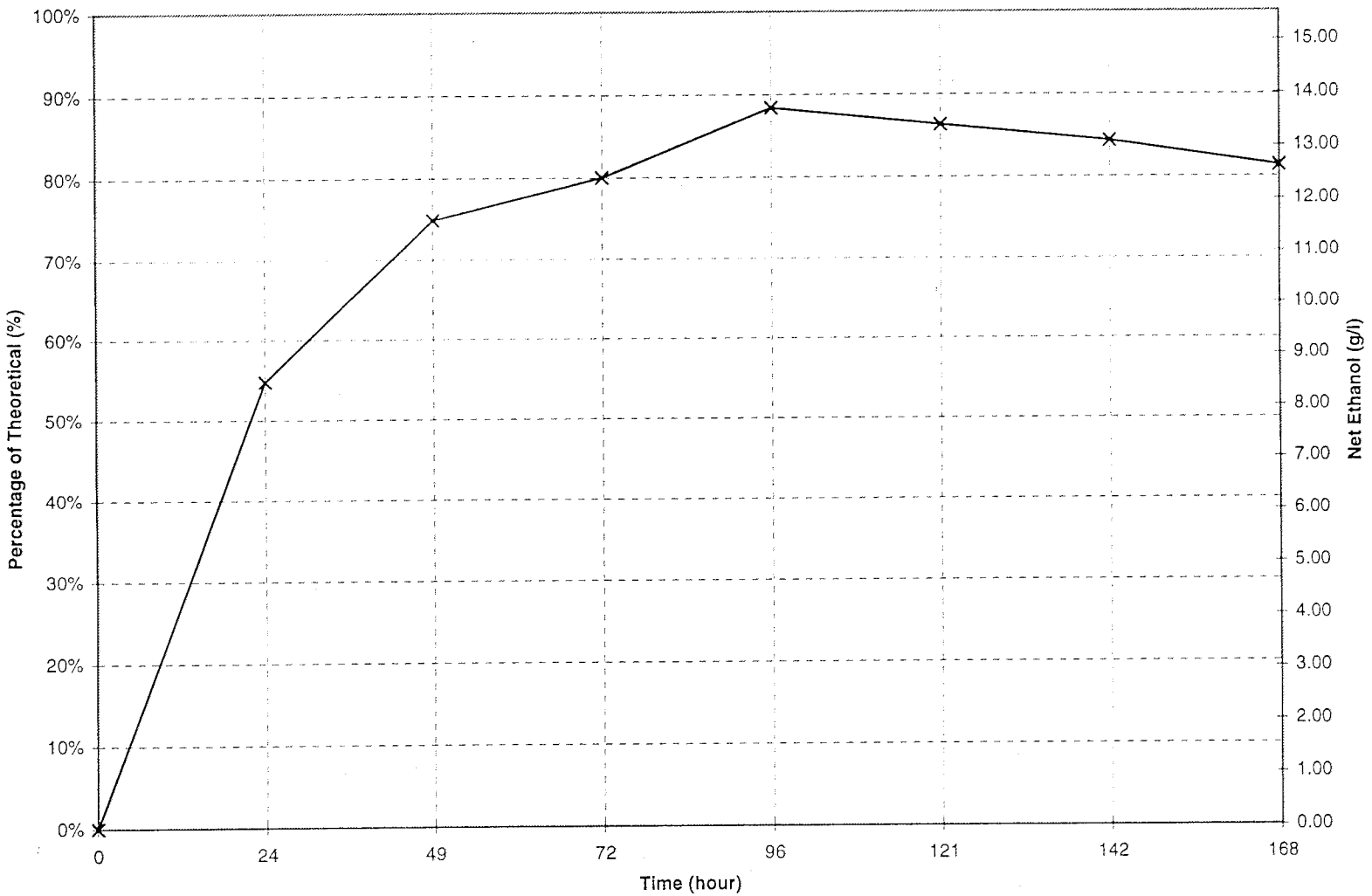

Figure 12. Net ethanol production in SSF of switchgrass reaction 3

Table 15. Products in SSF of switchgrass Reaction 10

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Acetic Acid HPLC	Lactic Acid HPLC	Glycerol HPLC
0	0	1	2.36	1.98	0.38	0.74	0.00	0.11	0.28	0.26	0.14
		2	1.49	1.34	0.27	0.52	0.00	0.20	0.23	0.20	0.17
24	23.5	1	12.43	12.30	0.71	0.00	7.96	1.94	0.25	0.22	0.31
		2	14.58	12.10	0.69	0.00	8.93	2.18	0.86	0.26	0.38
48	49.5	1	22.14	18.35	1.15	3.32	5.47	3.60	0.32	0.29	0.00
		2	25.09	18.85	0.66	1.82	5.46	3.87	0.37	0.28	1.19
72	71.7	1	19.33	17.00	4.06	9.83	4.38	3.47	0.32	0.29	1.20
		2	22.87	19.60	2.49	6.96	3.57	3.90	0.34	0.32	1.35
96	95.8	1	18.26	19.35	6.04	14.78	3.98	4.38	0.32	0.30	1.14
		2	19.90	21.55	4.42	11.35	2.94	4.36	0.42	0.52	1.29
120	120.6	1	18.42	18.10	7.77	~~17.94~~	3.80	4.38	0.41	0.48	1.28
		2	17.41	20.00	6.44	~~17.94~~	2.29	3.43	0.31	1.03	0.95
144	142.0	1	22.10	17.93	8.48	26.69	4.48	4.92	0.49	0.35	1.48
		2	22.95	20.13	6.11	18.94	3.19	4.47	0.47	0.33	1.36
168	168.0	1	16.79	17.20	10.50	21.04	2.69	3.65	0.41	0.29	1.05
		2	27.43	18.70	7.43	26.84	3.78	5.86	0.70	0.80	1.91

Strikethrough text is used when data was missing or anomalous and duplicate data was used for calculation purposes

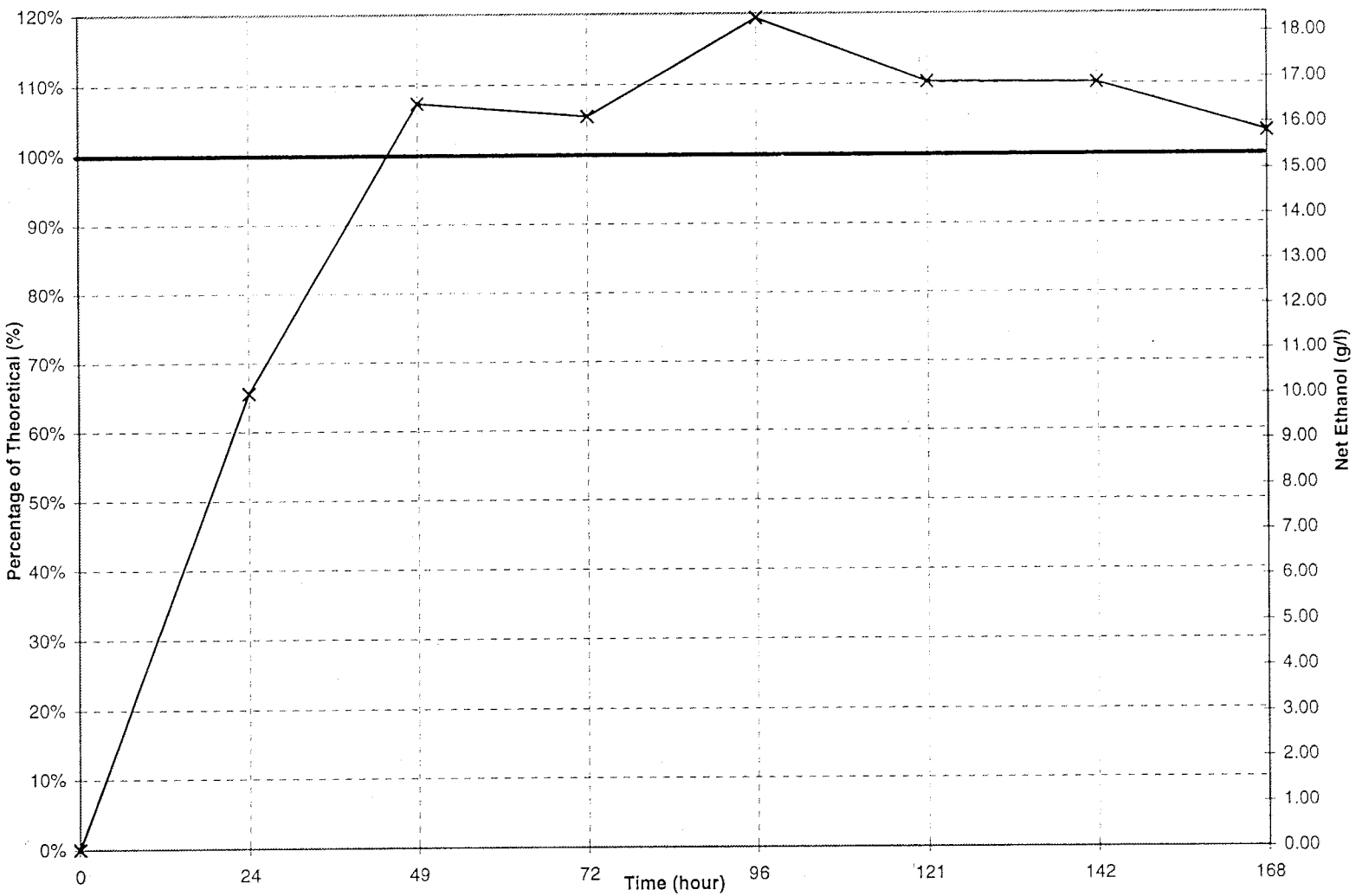

Figure 13. Net ethanol production in SSF of switchgrass reaction 10

The average percent of glycan conversion (fermentable hexosans) of 119.4% of 100 percentage of theoretical conversion in Table 13, also shown in Figure 13, indicated a serious error in experimental results. To determine the source of the error the results obtained were first carefully reviewed. It was highly improbable that the error would be due to experimental procedure. The results obtained in both Reactions 3 and 10 provided the QAQC that essentially eliminated an error in procedure.

A logical source of error could be due to the analyst following a specific analytical procedure. Since the error was duplicated it did not appear to be random. The original data recorded in Record Books kept for both SSF Reactions 3 and 10 were reviewed but no definitive explanation of this result was found.

The possibility that a fraction in the extractives of switchgrass might account for this high percentage of theoretical yield was considered. As noted below, the % of theoretical yield obtained for the switchgrass feedstock was higher than would be expected for wood such as hybrid poplar. Owing to the large difference in glycan contents between Reaction 3 and Reaction 10 residues the sample sizes taken to give 3.0 g of glucan for SSF varied widely. Reaction 10 sample weight (OD basis) was twice that of Reaction 3. An error in the glucan analysis of Reaction 10 or a lack of a representative sample in either the HPLC analysis or the selection of material for SSF could explain the high yield of ethanol.

The data for net ethanol production given in Table 13 summarized the results given in **Table 16** and **Figure 14** for SSF of -3/8 inch, -40 mesh, unextracted switchgrass feedstock.

In Table 16 it is shown by YSI analysis that ethanol production in the SSF reaction reached a maximum in 96 hours for both samples 1 and 2. As shown in Table 13 this amounted to a conversion to ethanol of 4.46 g/l; i.e., 25% of theoretical conversion. This was considered significant since it was substantially higher than the 8% theoretical conversion of hybrid poplar feedstock shown in Table 33 and Figure 21 of the Annual Report (Brink, 1995).

The good reproducibility of ethanol by YSI with unextracted and unhydrolyzed switchgrass is compatible with the presence of a material in the extractives that is hydrolyzed by enzymes and fermented to ethanol. The high percentage of theoretical yield occurred in the first sample taken at 23.5 hours. The yield for each time interval is a small incremental addition to the yield in the previous interval.

Thus, the substantially higher average percentage of theoretical conversion of unextracted switchgrass feedstock, 25.2%, at 96 hours in Table 13 compared to the average percentage of theoretical conversion of hybrid poplar feedstock, 8.1% at 96 hours demonstrates the presence of a fermentable component in the extractives and possibly a greater accessibility of cellulose (total glycans) in untreated switchgrass as compared to hybrid poplar.

The data for ethanol production given in Table 13 summarized the results given in **Table 17** and **Figure 15** for SSF of alpha cellulose (Sigma C8002).

Table 16. Products in SSF of -3/8 Inch, -40 Mesh, Unextracted Switchgrass Feedstock

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Acetic Acid HPLC	Lactic Acid HPLC	Glycerol HPLC
0	0	1	2.25	2.32	1.67	1.15	0.37	2.68	0.25	0.18	0.14
		2	2.60	2.07	2.75	1.15	0.42	2.78	0.31	0.22	0.20
24	23.5	1	5.26	6.92	0.10	0.00	0.33	1.12	0.37	0.08	0.24
		2	3.73	6.20	0.10	0.00	0.71	0.80	0.23	0.08	0.11
48	49.5	1	5.53	7.20	0.08	0.00	0.71	0.92	0.41	0.08	0.24
		2	4.74	7.57	0.05	0.00	0.59	1.09	0.36	0.08	0.18
72	71.7	1	7.66	7.20	0.10	0.79	1.01	1.83	0.64	0.08	0.43
		2	7.64	7.40	0.11	0.72	0.99	1.76	0.62	0.16	0.40
96	95.8	1	7.47	8.00	0.13	0.80	0.21	2.25	0.59	0.16	0.37
		2	7.62	7.56	0.12	0.68	0.18	2.38	0.61	0.16	0.37
120	120.6	1	7.59	7.54	0.30	0.00	0.97	2.22	0.63	0.16	0.42
		2	7.83	8.11	0.21	0.00	0.98	2.19	0.65	0.17	0.42
144	142.0	1	9.16	6.53	0.28	2.07	1.25	2.52	0.80	0.17	0.51
		2	7.53	8.05	0.35	1.38	0.92	1.98	0.64	0.16	0.40
168	168.0	1	6.79	6.69	0.13	0.00	0.83	1.99	0.57	0.15	0.34
		2	7.79	7.68	0.66	0.00	0.98	2.34	0.67	0.17	0.44

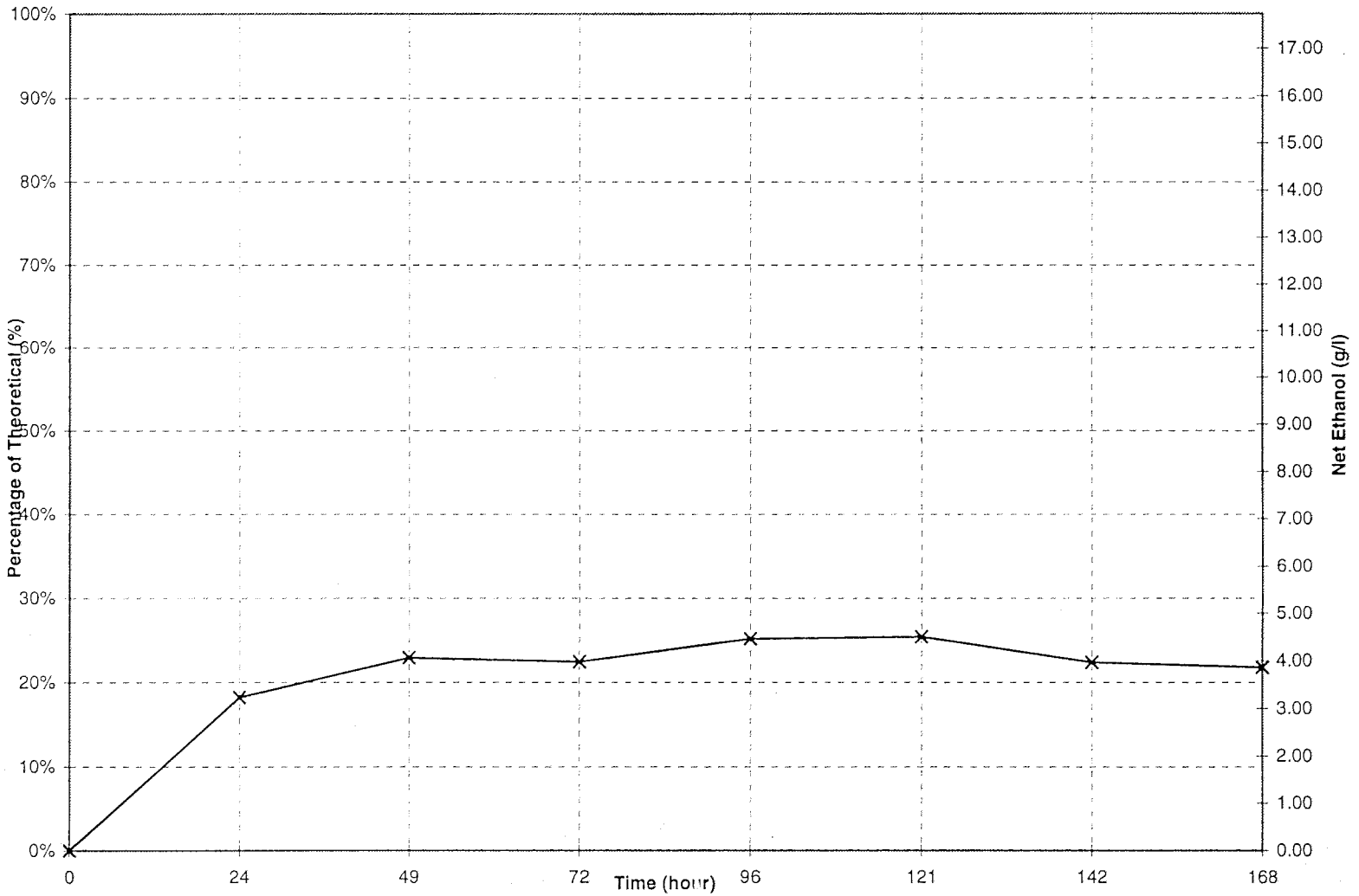

Figure 14. Net ethanol production in SSF of -3/8 inch, -40 mesh, unextracted switchgrass feedstock

Table 17. Products in SSF of α-cellulose (Sigma C8002)

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Acetic Acid HPLC	Lactic Acid HPLC	Glycerol HPLC
0	0	1	2.49	2.08	0.88	2.29	0.08	0.80	0.35	0.39	0.23
		2	3.02	2.05	0.60	3.10	0.32	0.38	0.38	0.32	0.19
24	23.5	1	9.82	9.18	0.22	0.00	0.31	0.20	0.42	1.10	0.00
		2	8.93	9.05	0.22	0.00	0.27	0.04	0.50	0.55	0.00
48	49.5	1	17.55	13.10	0.18	0.55	1.02	1.52	0.30	0.08	0.41
		2	12.23	12.50	0.19	0.42	0.66	1.03	0.00	0.08	0.21
72	71.7	1	24.42	15.20	0.12	1.16	1.28	1.90	0.41	0.08	0.73
		2	14.91	15.30	0.13	0.42	0.58	0.88	0.22	0.08	0.30
96	95.8	1	16.49	15.75	0.06	0.53	0.29	2.11	0.23	0.08	0.51
		2	16.48	16.30	0.05	0.00	0.23	1.93	0.21	0.08	0.37
120	120.6	1	17.52	17.45	0.07	0.00	0.58	1.46	0.25	0.17	0.66
		2	15.54	17.75	0.04	0.00	0.38	0.80	0.19	0.08	0.31
144	142.0	1	18.28	17.27	0.04	0.05	0.46	0.99	0.24	0.18	0.69
		2	18.48	17.93	0.03	0.04	0.43	0.65	0.26	0.08	0.41
168	168.0	1	17.23	18.90	0.06	0.58	0.43	1.07	0.27	0.34	0.74
		2	13.99	19.40	0.03	0.57	0.45	0.63	0.20	0.08	0.28

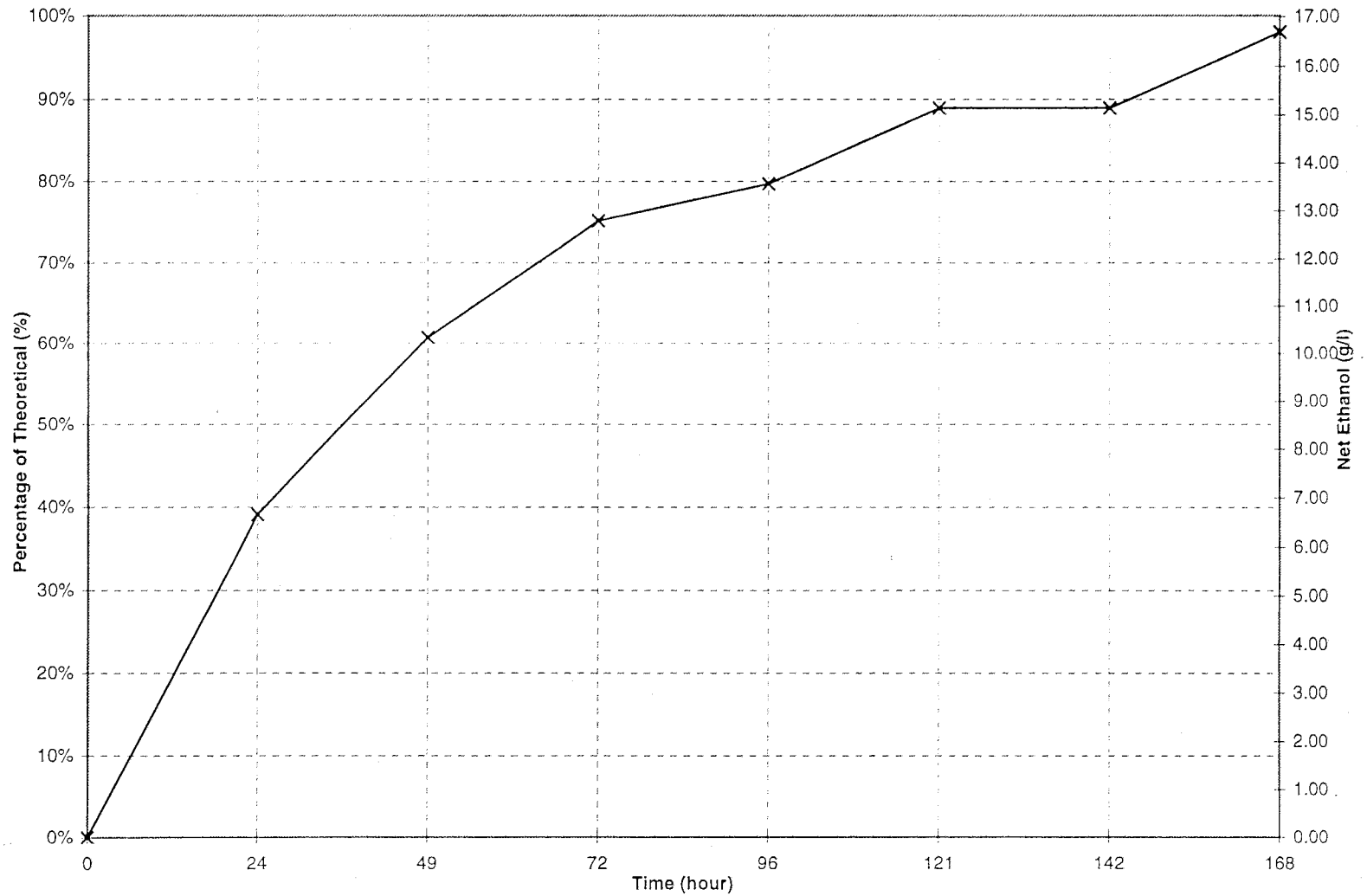

Figure 15. Net ethanol production in SSF of a-cellulose (Sigma C8002)

In Table 17 it is shown by YSI analysis that ethanol production in the SSF reaction reached an average (samples 1 and 2) concentration (g/l) of ethanol in 96 hours of 16.02 compared to switchgrass reaction 3 of 16.2 shown in Table 14. In Table 13 this amounted to a % theoretical conversion of glycans of 79.7 % compared to 89.5% in switchgrass reaction 3. It was recognized that the rate of conversion of alpha-cellulose is slower due to the increase of hydrogen bonding during the preparation of the alpha-cellulose. It was also recognized that the % of theoretical conversion finally reached in alpha cellulose will be comparable to that obtained with dilute acid pretreated residues such as switchgrass reaction 3. This was observed in Table 13 results for alpha cellulose in which the percentage of theoretical yield in 121 and 142 hours was 88.9%. The increase observed for 168 hours was considered to be due to an experimental error. The percentage of theoretical conversion reached with this same alpha-cellulose and reported in Table 34a and Figure 22 of the Annual Report (Brink, 1995) gave a maximum in the average (2 samples) % of theoretical conversion of 85.5% in 141 hours. These two plots, run 18 months apart, are very similar.

The net ethanol production as an average of the two samples of the switchgrass materials and alpha-cellulose subjected to SSF is given in **Figure 16**.

These results presented in this one figure, Net ethanol in SSF of switchgrass and alpha-cellulose (Sigma C8002) (average of 2 samples), support the discussion presented above on each of these materials and show the range of ethanol concentrations encountered.

The products of SSF, including ethanol, glucose determined by YSI, and others products determined by HPLC are shown in Figures 17 - 20. These included glucose + mannose, cellobiose, xylose + galactose, acetic acid, lactic acid, and glycerol. The products for switchgrass Reaction 3 are summarized in Table 14 and **Figure 17**. Relatively low concentrations of metabolic products and intermediates were noted in this fermentation. A peak for xylose plus galactose confirms the presence of xylose in the hydrolyzed switchgrass residue. Since galactose was not present in this residue, the relatively constant 1.5 - 2.5 g/l can be attributed to xylose. The highest concentration of cellobiose intermediate was observed at 23.5 hours (2.02 g/l, sample 2-1). This is consistent with the results obtained with hybrid poplar residues which showed a maximum cellobiose concentration of 2 - 3 g/l at the first sampling time of approximately 24 hours. Unlike hybrid poplar, however, where cellobiose decreased to very low levels by 96 hours, the switchgrass Reaction 3 cellobiose remained at 0.4 g/l even at 168 hours. Only hybrid poplar duplicate 4-2 showed a similar result. This may result from the cellobiohydrolase component of the enzyme being inhibited for some reason and not converting cellobiose to glucose. The very low levels of glucose in the later Reaction 3 samples indicate the yeast was still converting the glucose to ethanol as it was produced.

The products for switchgrass reaction 10 are summarized in Table 15 and **Figure 18**.

It was previously noted that the net ethanol concentration in this SSF reaction exceeded that which is theoretically possible. Figure 18 shows the presence of significant concentrations of both cellobiose and glucose which means that the potential ethanol would have been even higher than that observed.

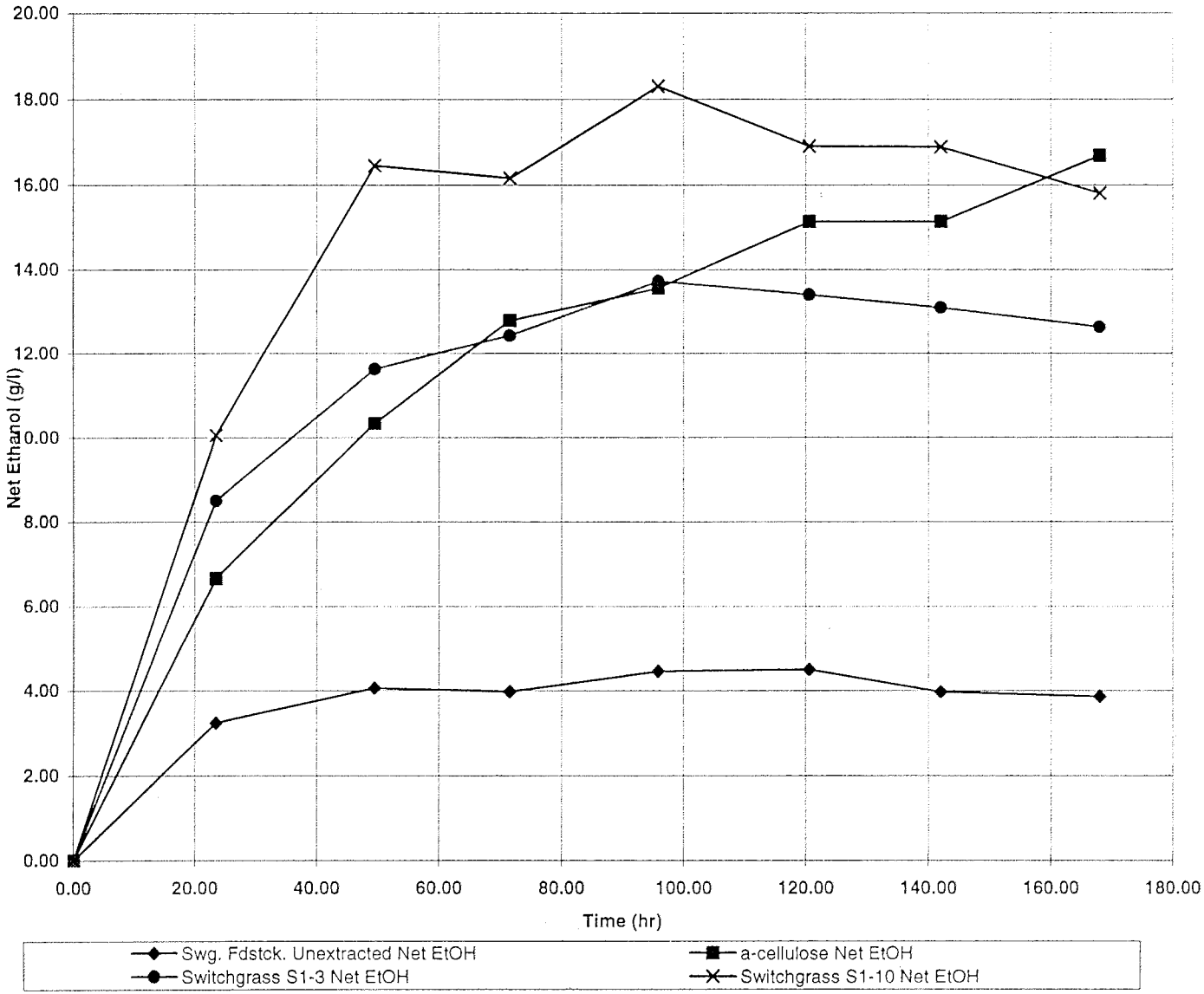

Figure 16. Net ethanol in SSF of switchgrass and α-cellulose (Sigma C8002) (average of 2 samples)

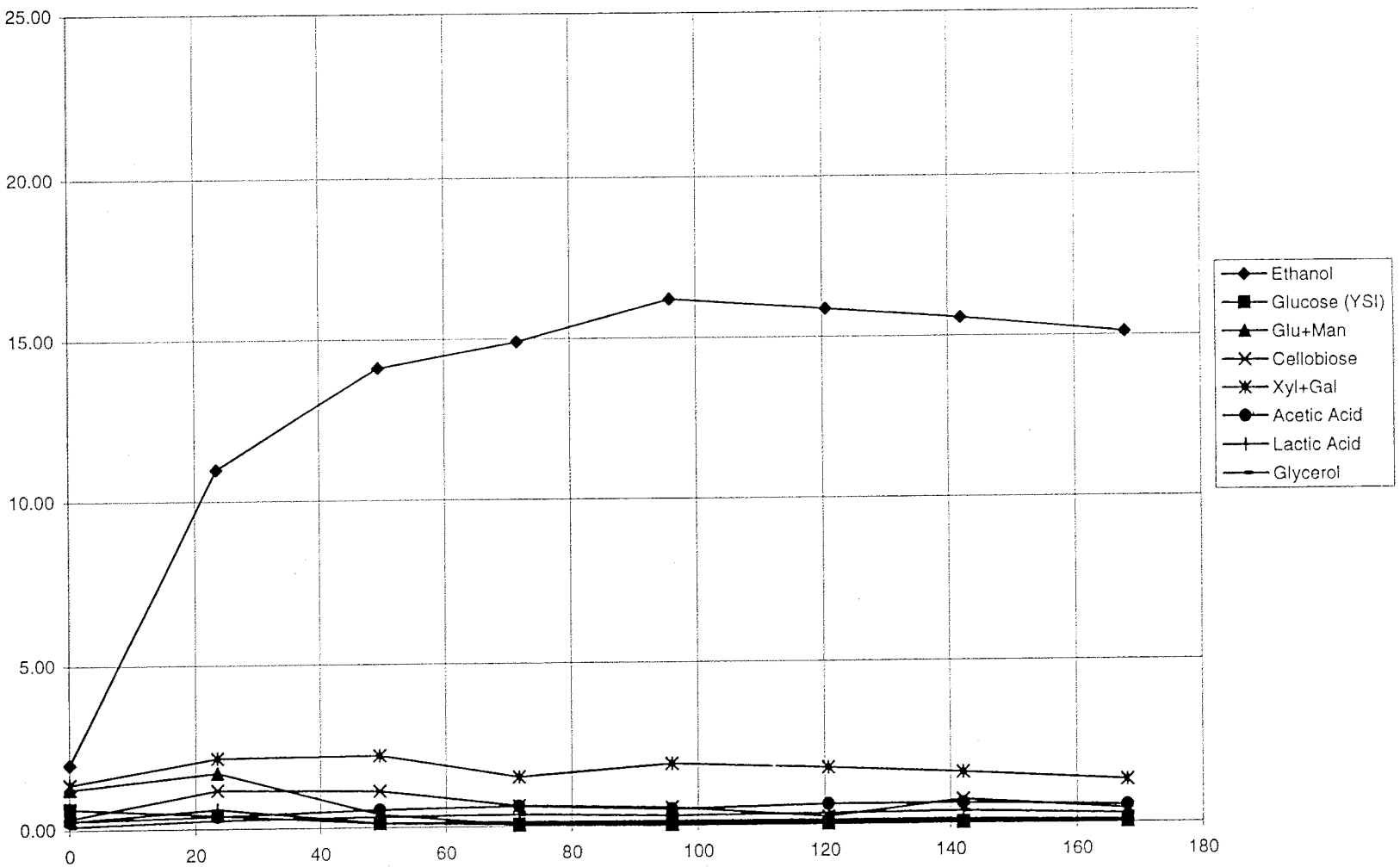

Figure 17. Products in SSF of switchgrass reaction 3

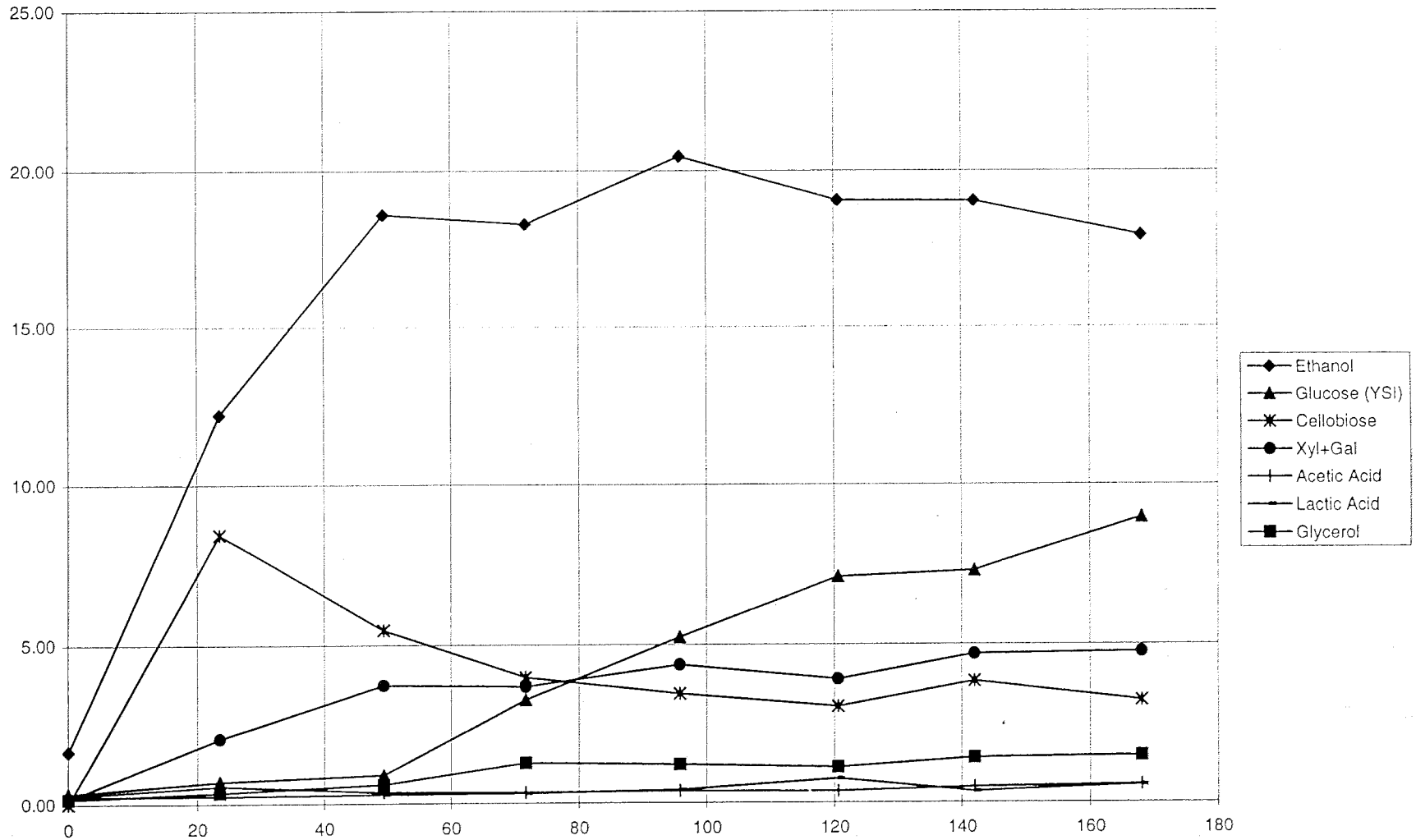

Figure 18. Products in SSF of switchgrass reaction 10

Cellobiose concentration was highest at 23.5 hours but at levels twice that observed in any other switchgrass or hybrid poplar SSF reaction. Cellobiose concentration decreased to approximately 3.5 g/l by 96 hours where it remained for the duration of the reaction. Ethanol production tended to level off after 49.5 hours, the same time that glucose concentration started to rise, ultimately reaching 9 at 168 hours. One possible factor is the relatively large amount of Reaction 10 residue present. The cellulase enzyme production of cellobiose may have been retarded by adsorption on the biomass surface. The cellobiohydrolase continued to function as evidenced by the increasing glucose concentration. At this time the yeast ceased to ferment the glucose and the ethanol concentration leveled off. This may have occurred by lack of yeast nutrients, coproduct inhibition and cell death. A definitive explanation is not available at this time.

The products for switchgrass unextracted feedstock are summarized in Table 16 and **Figure 19**.

The products for alpha-cellulose (Sigma G8002) are summarized in Table 17 and **Figure 20**.

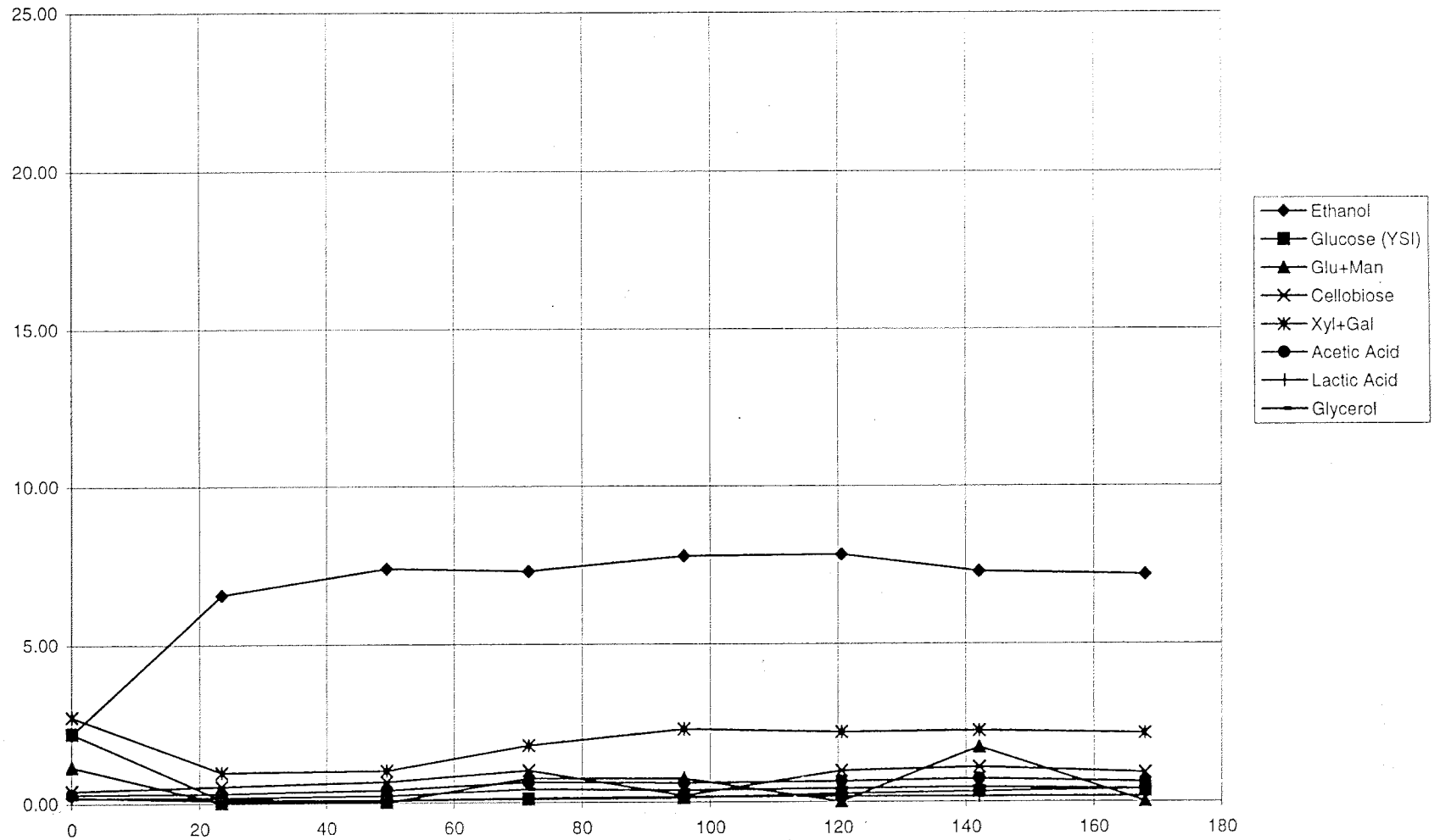

Figure 19. Products in SSF of -3/8 inch, -40 mesh, unextracted switchgrass feedstock

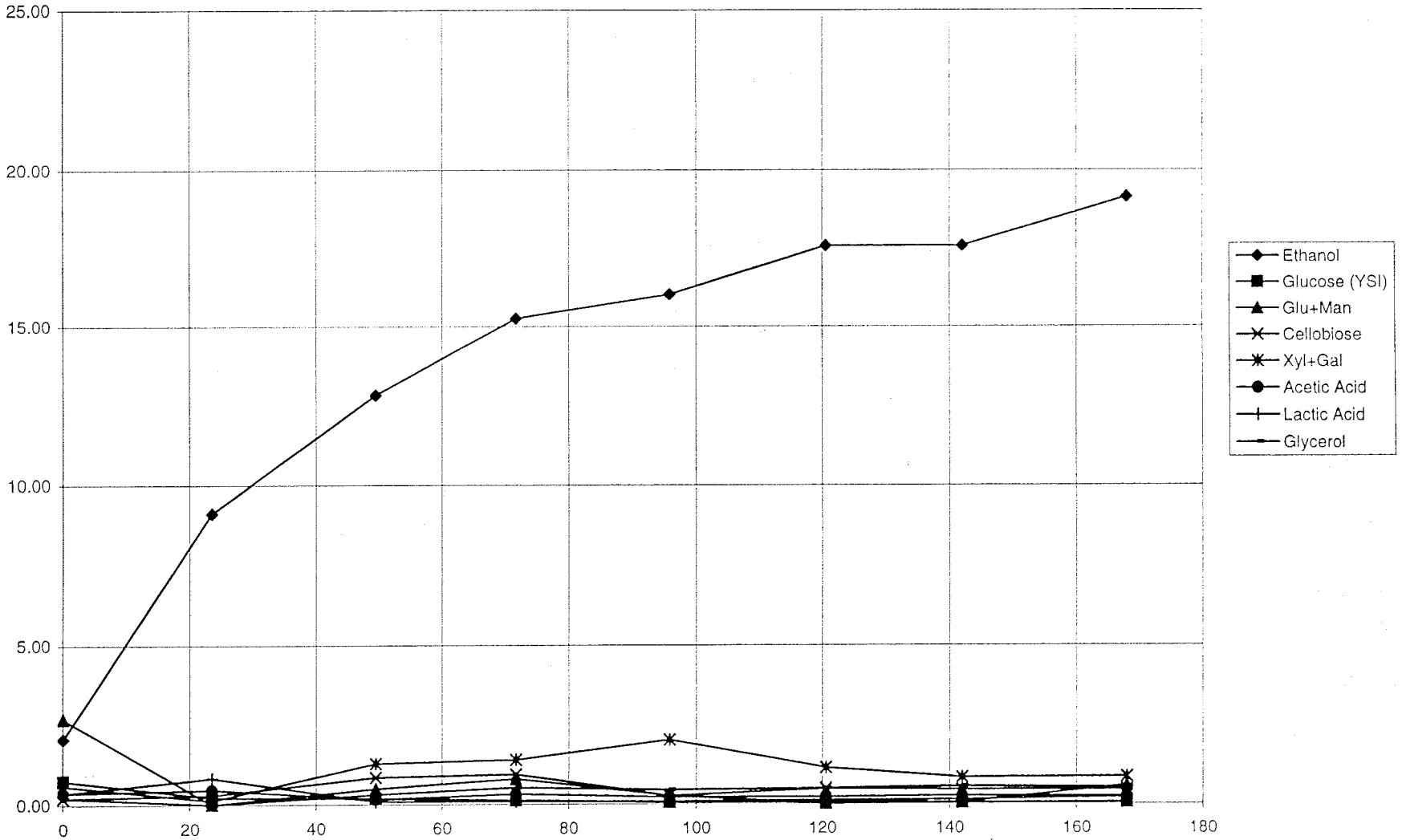

Figure 20. Products in SSF of α-cellulose (Sigma C8002)

5. Pretreatment Liquor Fermentation

The pretreatment liquors from switchgrass steady-state intervals were saved and retained as described in Section 3.3.3, Storage of Pretreatment Products. The switchgrass pretreatment liquor from Reaction 3 was retained at pH 1.4 in cold storage for 3 months and analyzed within one month of use in *Pichia stipitis* fermentation. The liquor from switchgrass reaction 10 was prepared and retained at pH 1.4 in cold storage for less than a month before being analyzed and used in *P. stipitis* fermentation. The hybrid poplar pretreatment liquor from Reactions 1 and 3 were prepared six months before being analyzed and used in *P. stipitis* fermentations. The analyses were completed within a week of the start of fermentation. In Section 4.2.3.8, Pretreatment Liquor Composition, and Section 4.2.4 of the Annual Report (Brink, 1995) the procedures and problems involved with preparation and storage of hybrid poplar pretreatment liquor were described in detail. This information is included in the Final Report by reference.

5.1 Dry Cell Mass Determination in Pichia Stipitis Fermentation

Dry cell mass was determined following the NREL protocol discussed in Section 2, Task 2. Samples for analysis were taken at nominal 24-hour intervals from 0 hours to 96 hours. The results are given in Sections 5.1.1 through 5.1.4.

5.1.1 Hybrid Poplar Reaction 1

Dry cell mass in Pichia stipitis fermentation of hybrid poplar Reaction 1 are shown in **Table 18** and **Figure 21** for 0%, 40% and 80% hydrolysates. The growth of the yeast in 44 hours with 0% hydrolysate was rapid reaching a maximum of 12 g/l. The growth in a medium containing 40% hydrolysate was 9.2 g/l; whereas, the growth in 80% hydrolysate was least at 7.7 g/l. Thus, the hydrolysate had an inhibitory effect on yeast growth.

Except for the zero time samples, the dry cell mass values were determined spectrophotometrically on a 0.5 ml sample taken in addition to a 3.0-ml sample for HPLC analysis at each 24-hour interval. The small sample was diluted by a factor of 100, the absorbance at 600nm was measured and the dry cell mass determined from a calibration curve (Figure 1). The large dilution factor may account for the apparent randomness seen in Figure 21.

5.1.2 Hybrid Poplar Reaction 3

Dry cell mass in *Pichia stipitis* Fermentation of hybrid poplar Reaction 3 are shown in **Table 19** and **Figure 22** for 0%, 40%, and 80% hydrolysates. Results were different than those observed for hybrid poplar Reaction 1 in that the 20 hour growth for 0 g/l hydrolysate medium, 8.7 g/l, was almost at the maximum reached in 96 hours, 9.4 g/l. Growth in both 40% and 80% hydrolysate media had essentially reached a maximum in 42 hours that was approximately 15% and 10% higher than that reached in 20 hours, respectively. Again inhibitory effect on yeast growth was observed.

It can be noted that cell concentration in both Reactions 3 and 1 with zero hydrolysate doubled or almost doubled over the fermentation period. With both 40% and 80% hydrolysate cell growth was more modest.

Table 18. Dry Cell Mass in Pichia stipitis Fermentation of Hybrid Poplar Reaction 1, 0%-80% Hydrolyzate

		Dry Cell Mass (g/l)		
Time Nominal (hours)	Time Actual (hours)	0% Hydrol.	40% Hydrol.	80% Hydrol.
0	0	5.59	5.59	5.59
24	20.6	8.29	5.39	3.76
48	43.8	11.93	9.27	7.72
72	67.3	10.75	6.19	4.58
96	90.5	11.48	7.78	6.94

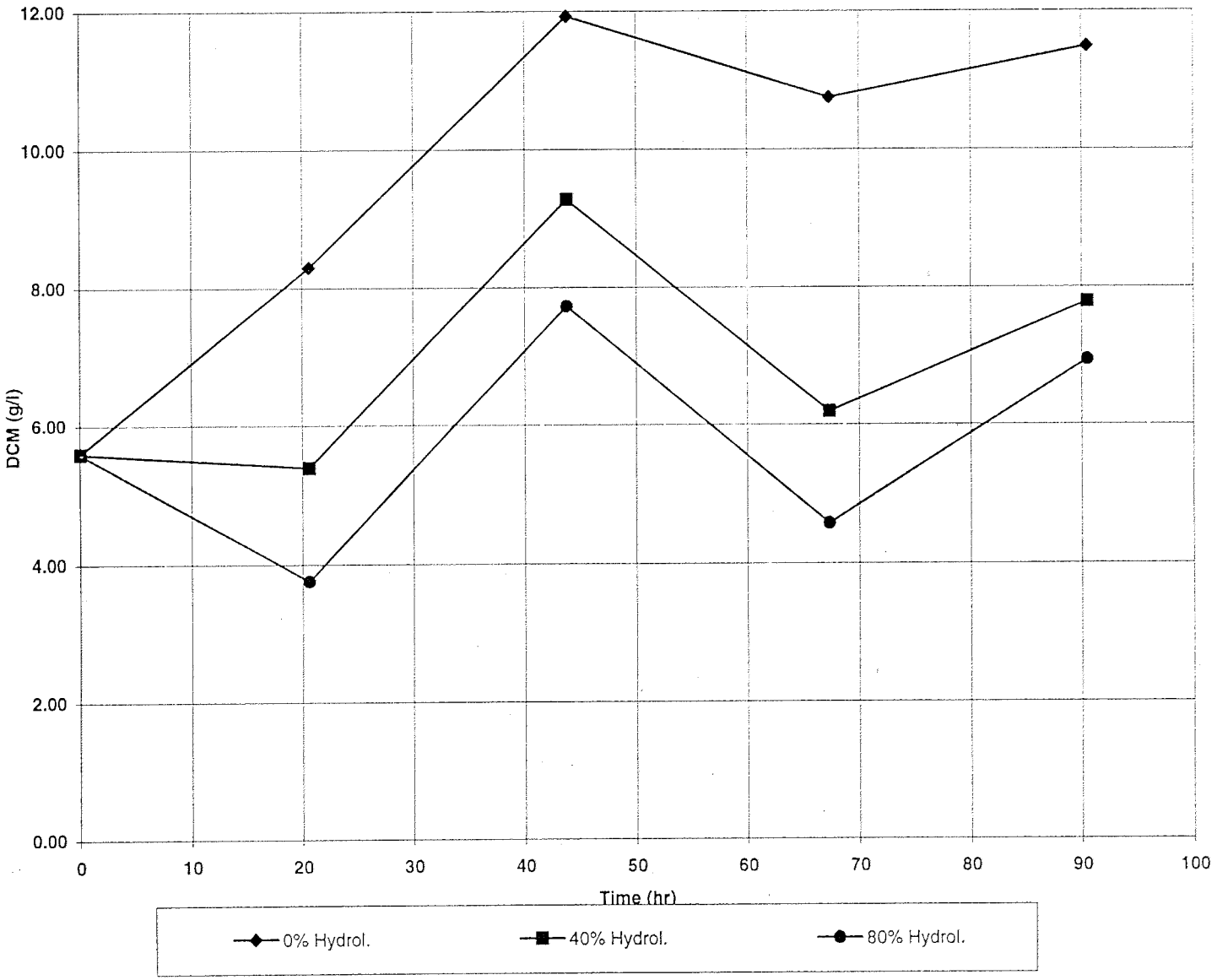

Figure 21. Dry cell mass in Pichia stipitis fermentation of hybrid poplar reaction 1, 0%-80% hydrolyzate

Table 19. Dry Cell Mass in Pichia stipitis Fermentation in Hybrid Poplar Reaction 3, 0%-80% Hydrolyzate

		Dry Cell Mass (g/l)		
Time Nominal (hours)	Time Actual (hours)	0% Hydrol.	40% Hydrol.	80% Hydrol.
0	0	5.01	5.01	5.01
24	20.2	8.72		
48	42.0	9.17	6.91	6.26
72	65.1			
96	87.0	9.47	6.71	7.33

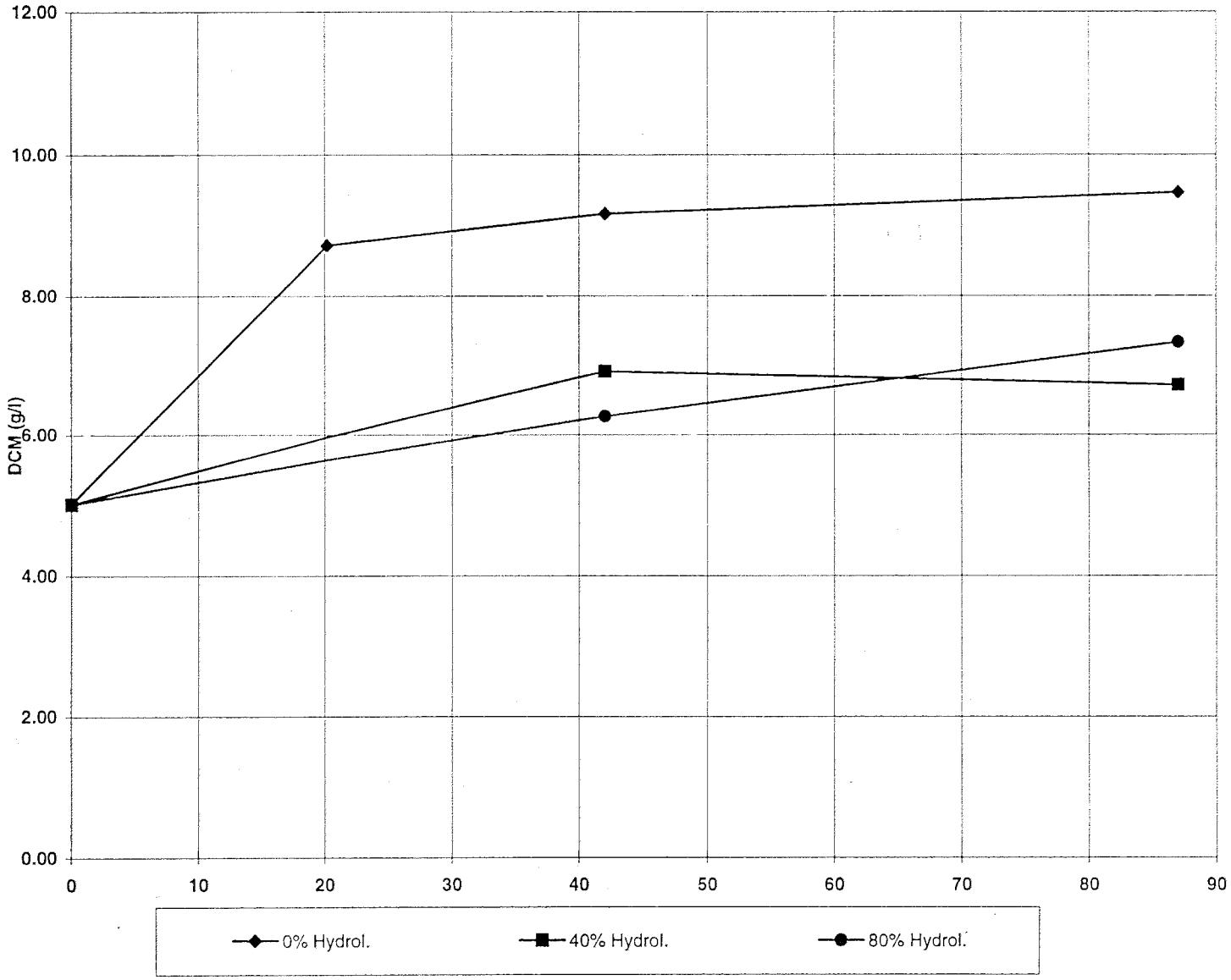

Figure 22. Dry cell mass in Pichia stipitis fermentation of hybrid poplar reaction 3, 0%-80% hydrolyzate

5.1.3 Switchgrass Reaction 3

The *P. stipitis* fermentation of two switchgrass hydrolysates was carried out in one experiment. In addition to 0%, 40%, and 80% hydrolysates a 90% hydrolysate (in duplicate) was used. In order to maintain a total volume of 100 ml the 10-ml of yeast extract added to the other flasks was omitted and the required xylose was added as crystalline xylose.

Dry cell mass in *Pichia stipitis* fermentation of switchgrass Reaction 3 are shown in **Table 20** and **Figure 23** for 0%, 40%, 80% and 90% hydrolysate. In switchgrass Reaction 3 the 40% hydrolysate was similar but about 7% higher than that of 0% hydrolysate. The growth at both 80% and 90% hydrolysate in the medium was essentially inhibited.

5.1.4 Switchgrass Reaction 10

Dry cell mass in *Pichia stipitis* fermentation of switchgrass Reaction 10 are shown in **Table 21** and **Figure 24** for 0%, 40%, 80% and 90% hydrolysate. The growth of yeast in the medium at 0% hydrolysate increased to 92 hours. Unlike switchgrass Reaction 3, the growth in the medium containing 40% hydrolysate was lower at 22 hours than that of the medium containing 0% hydrolysate. The growth in the media containing 80% and 90% hydrolysates was essentially inhibited increasing to only about 5 g/l.

5.2 Net Ethanol Production

Net ethanol production in *Pichia stipitis* fermentation are shown in Tables 22 - 25 and Figures 25 - 41.

5.2.1 Hybrid Poplar Reaction 1

Ethanol production in *Pichia stipitis* fermentation of hybrid poplar Reaction 1 hydrolysate is shown in **Table 22**. In **Figure 25** net ethanol production in 0% hydrolysate reached a maximum of 104% of the percentage of theoretical in 20.6 hours or less. This demonstrated the robust activity of the yeast. This was also borne out in the dry cell mass results shown in Table 18 and Figure 21 in which the maximum yield, 11.93 g/l, was obtained in 43.8 hours. As shown below these results were better than those in the presence of hydrolysate. The net ethanol yield with 40% hydrolyzate was still very good, giving 97% of theoretical ethanol yield. Although the yeast showed some effect of inhibition virtually all fermentables present were eventually converted to ethanol. The maximum for both 40% and 80% hydrolysate occurs at the 43.8 hour sampling point resulting in lower ethanol volume productivity. The 80% hydrolysate fermentation gave an average net ethanol yield of 0.44 g/g representing 84% of the theoretical. These results suggest that acclimation of the yeast to this hydrolysate may produce even better results.

The ethanol values used in the two hybrid poplar runs, Reactions 1 and 3, are based on HPLC. At the time the YSI analyzer was not available. The YSI ethanol values shown for Reaction 1 in Tables 26 -28 were obtained from stored samples taken well after the fermentation experiment and were not considered to be as reliable as the HPLC analyses done while the fermentation was underway. Figures 26 and 27 show the net ethanol production in g/l for 40% and 80% hydrolysates, respectively.

Table 20. Dry Cell Mass in Pichia stipitis Fermentation in Switchgrass Reaction 3, 0%-90% Hydrolyzate

Time Nominal (hours)	Time Actual (hours)	Dry Cell Mass (g/l) 0% Hydrol.	40% Hydrol.	80% Hydrol.	90% Hydrol.
0	0	3.69	3.69	3.69	3.69
24	22.2	7.63	9.25	3.92	3.39
48	45.3	9.08	9.59	5.28	4.37
72	68.8	8.36	9.15	4.67	3.37
96	92.4	10.55	11.37	5.27	3.92

Table 21. Dry Cell Mass in Pichia stipitis Fermentation in Switchgrass Reaction 10, 0%-90% Hydrolyzate

Time Nominal (hours)	Time Actual (hours)	Dry Cell Mass (g/l) 0% Hydrol.	40% Hydrol.	80% Hydrol.	90% Hydrol.
0	0	3.69	3.53	3.69	3.69
24	22.2	7.63	6.48	4.42	4.39
48	45.3	9.08	6.31	5.43	4.91
72	68.8	8.36	4.96	4.60	4.64
96	92.4	10.55	4.90	4.83	4.94

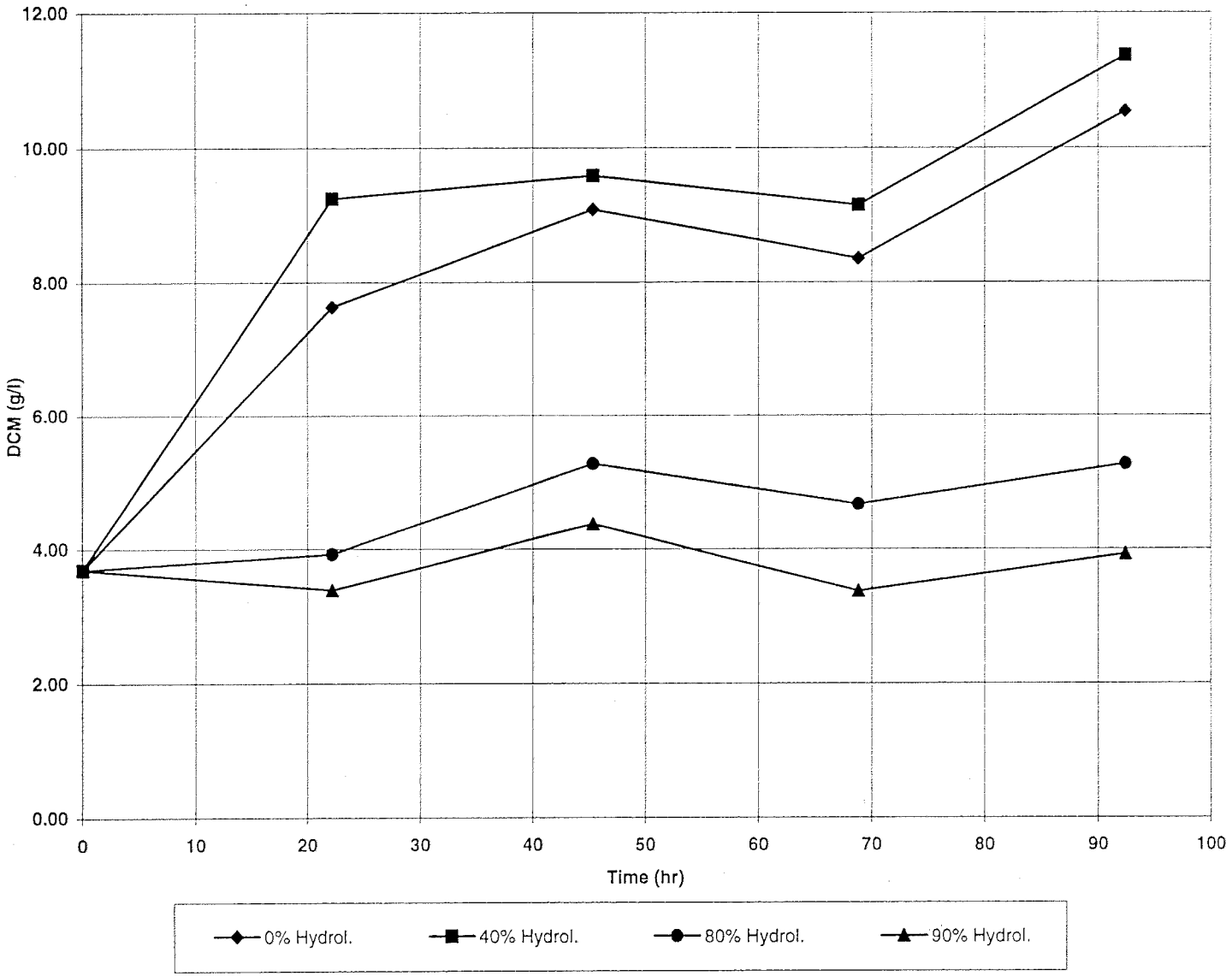

Figure 23. Dry cell mass in Pichia stipitis fermentation of switchgrass reaction 3, 0%-90% hydrolyzate

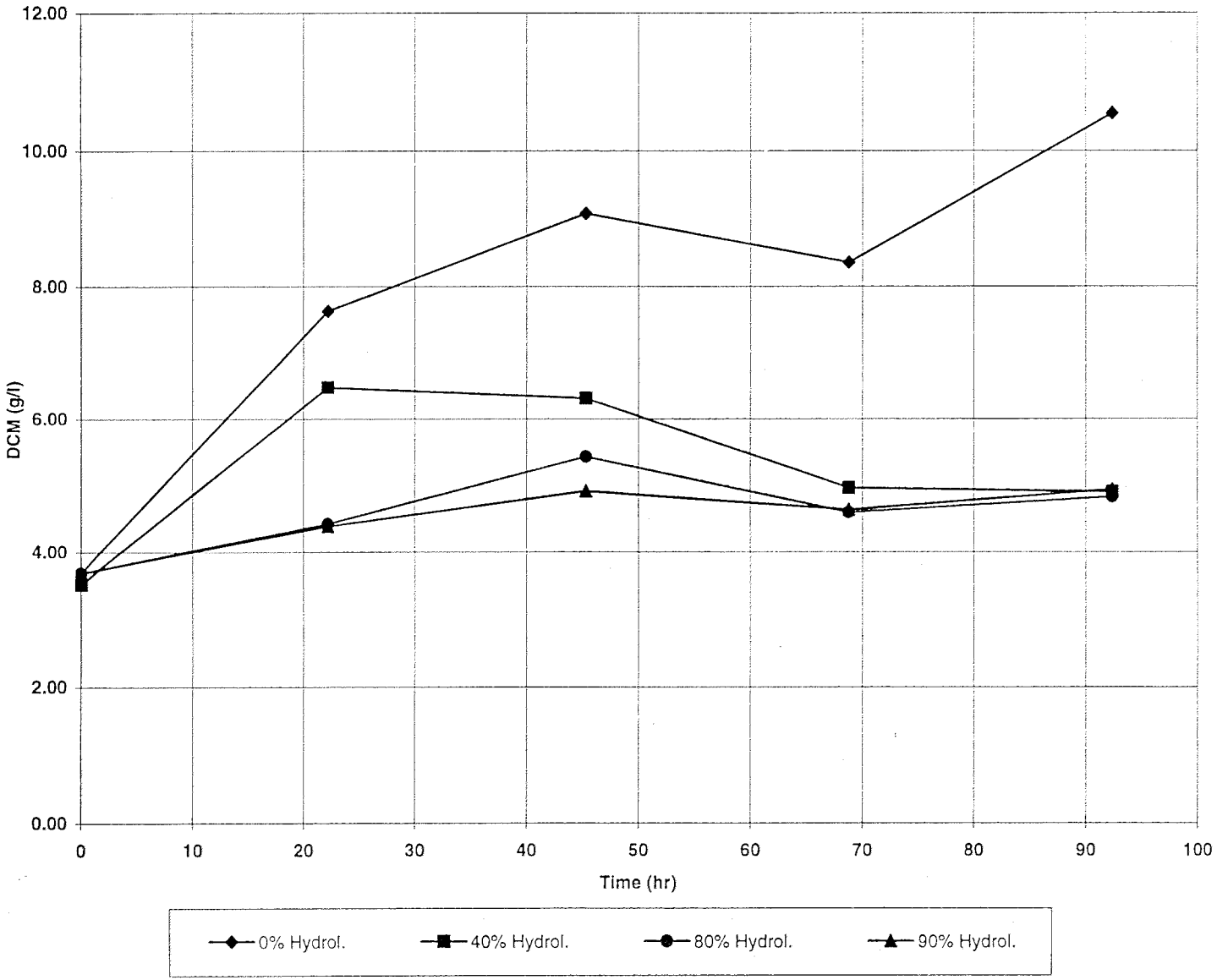

Figure 24. Dry cell mass in Pichia stipitis fermentation of switchgrass reaction 10, 0%-90% hydrolyzate

Table 22 Ethanol Yields in Pichia stipitis Fermentation of Hybrid Poplar Reaction 1, 0%-80% Hydrolyzate

Hydrolyzate, %	0	40	80
Time Zero Concentration, g/l			
Glucose+Mannose	0.00	1.15	2.52
Xylose+Galactose	24.21	30.00	34.60
Cellobiose	0.00	0.72	0.77
Ethanol	1.50	1.50	1.50
Max. Net Ethanol Yield*, g/g	0.53	0.49	0.44
Max. Net Ethanol Concentration, g/l	12.83	15.76	16.34
Time to Max. Net Ethanol, hours	20.60	43.80	43.80
Average Net Ethanol Volume Productivity, g/l/hr	0.62	0.36	0.37
Maximum Theoretical Ethanol*, g/l	12.37	16.28	19.38
Experimental Ethanol Yield as Percentage of Theoretical Max.	104%	97%	84%

* based on grams of total fermentable sugars present

Table 23 Ethanol Yields in Pichia stipitis Fermentation of Hybrid Poplar Reaction 3, 0%-80% Hydrolyzate

Hydrolyzate, %	0	40	80
Time Zero Concentration, g/l			
Glucose+Mannose	0.34	3.80	7.59
Xylose+Galactose	28.79	34.20	35.55
Cellobiose	0.38	1.45	1.44
Ethanol	0.55	0.55	0.55
Max. Net Ethanol Yield*, g/g	0.45	0.38	0.38
Max. Net Ethanol Concentration, g/l	13.15	14.90	17.04
Time to Max. Net Ethanol, hours	20.20	65.10	65.10
Average Net Ethanol Volume Productivity, g/l/hr	0.65	0.23	0.26
Maximum Theoretical Ethanol*, g/l	15.09	20.20	22.83
Experimental Ethanol Yield as Percentage of Theoretical Max.	87%	74%	75%

* based on grams of total fermentable sugars present

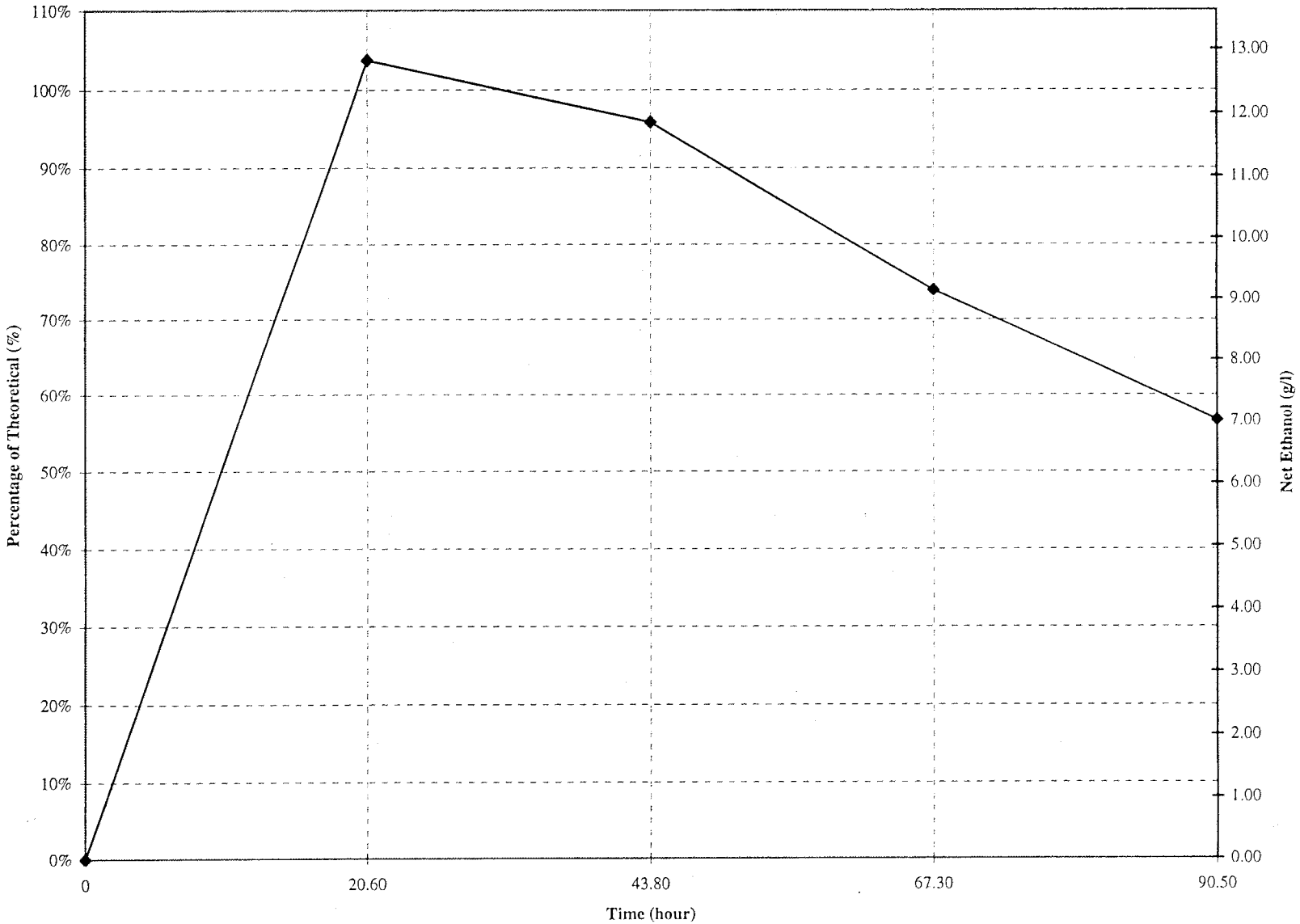

Figure 25 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 1, 0% hydrolysate.

Figure 28 shows all the data. The declining ethanol concentration after the maximum arises from the ability of *P. stipitis* to assimilate ethanol as the fermentable sugars are used up. This was observed in all *P. stipitis* fermentations.

5.2.2 Hybrid Poplar Reaction 3

Ethanol yield and productivity data from *P. stipitis* fermentation on Reaction 3 hydrolysate is shown in **Table 23** and net ethanol production versus time for 0%, 40%, and 80% hydrolysates are plotted in **Figures 29-31**. **Figure 32** shows all net ethanol values.

The Reaction 3 results are similar to those of Reaction 1 but with lower yields. Net ethanol yields for 0%, 40%, and 80% are 0.45, 0.38 and 0.38 g/g, respectively. Inhibition appears to be slightly greater than in Reaction 1. The slower rate of fermentation delayed the maximum ethanol to 65.1 hours for both the 40% and 80% hydrolysate samples resulting in lower ethanol volumetric productivity. The maximum net ethanol yields as a percent of theoretical are 87%, 74%, and 75%, respectively for 0%, 40%, and 80% hydrolysates. Chemical compositions of the fermentates are presented in Tables 29 - 31.

5.2.3 Switchgrass Reaction 3

The ethanol yield data from *Pichia stipitis* fermentation of switchgrass Reaction 3 hydrolysate is presented in **Table 24**. Plots of net ethanol concentration and theoretical yield are shown in **Figures 33-37**. The control fermentation containing no hydrolysate but added xylose solution gave 100% conversion to ethanol at the first sampling interval of 22 hours. The 40% hydrolysate was also fermented readily to give 92% of theoretical yield at the same time. The 80% hydrolysate was inhibitory to the yeast and the rate of fermentation was slow but ultimately by 92 hours 78% of the theoretical ethanol had been produced. The 90% hydrolysate sample, which contained no yeast extract was seriously inhibited giving only 21% of maximum possible ethanol.

5.2.4 Switchgrass Reaction 10

Ethanol yield data from *Pichia stipitis* fermentation of switchgrass Reaction 10 hydrolysate is shown in **Table 25**. Plots of net ethanol concentration and theoretical yield are shown in **Figures 38-41**. This hydrolysate was somewhat more inhibitory to *Pichia stipitis* but the overall ethanol yields were similar. The experimental ethanol yield of 40%, 80% and 90% hydrolysate were 87%, 70%and 58% respectively. The 40% hydrolysate didn't reach maximum ethanol yield until 45 hours in contrast to switchgrass Reaction 3, which peaked at 22 hours. The 80% hydrolysate shows the same pattern as switchgrass Reaction 3 and the two hybrid poplar hydrolysates of reduced rate of fermentation but eventually significant production of ethanol.

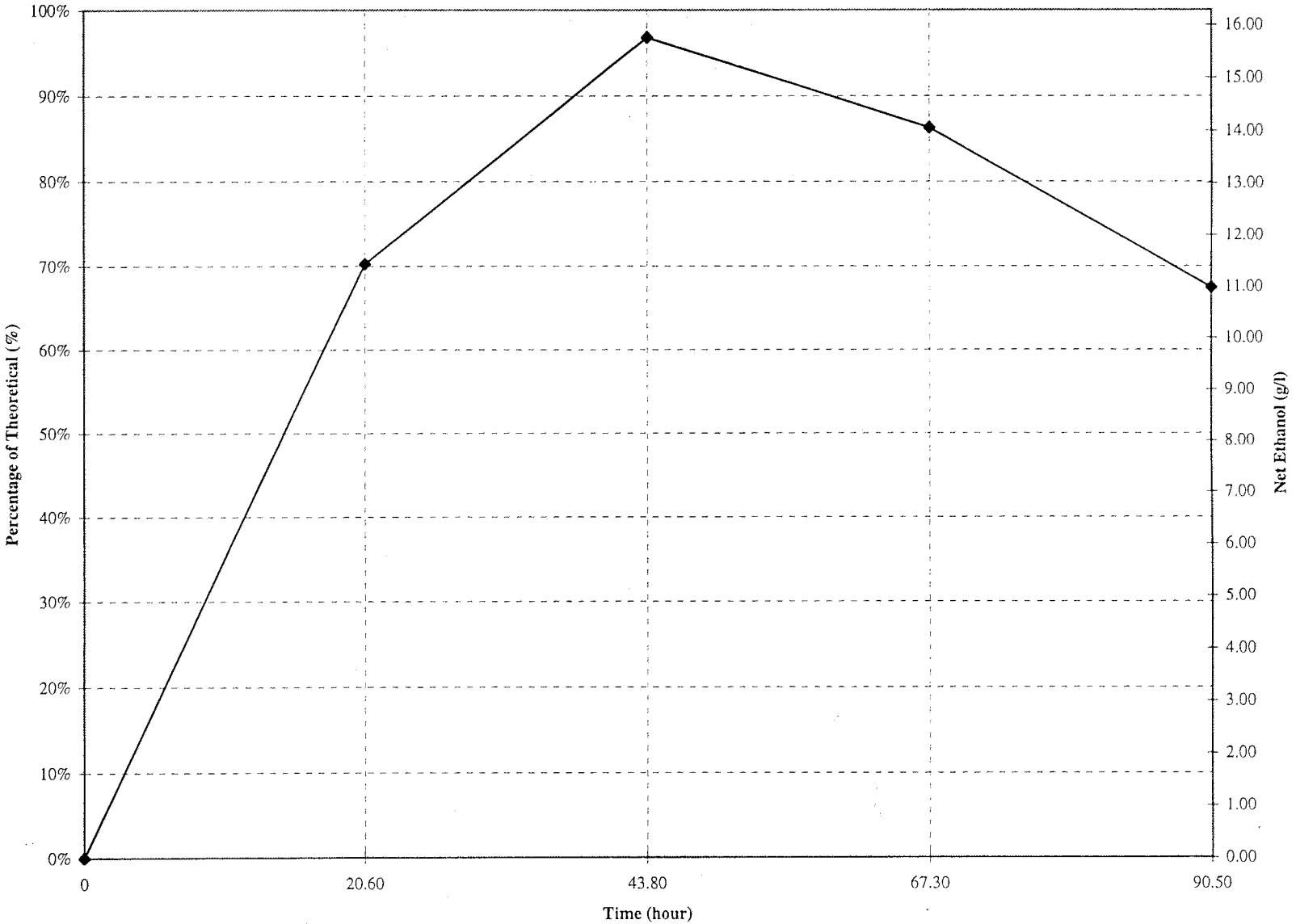

Figure 26 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 1, 40% hydrolysate.

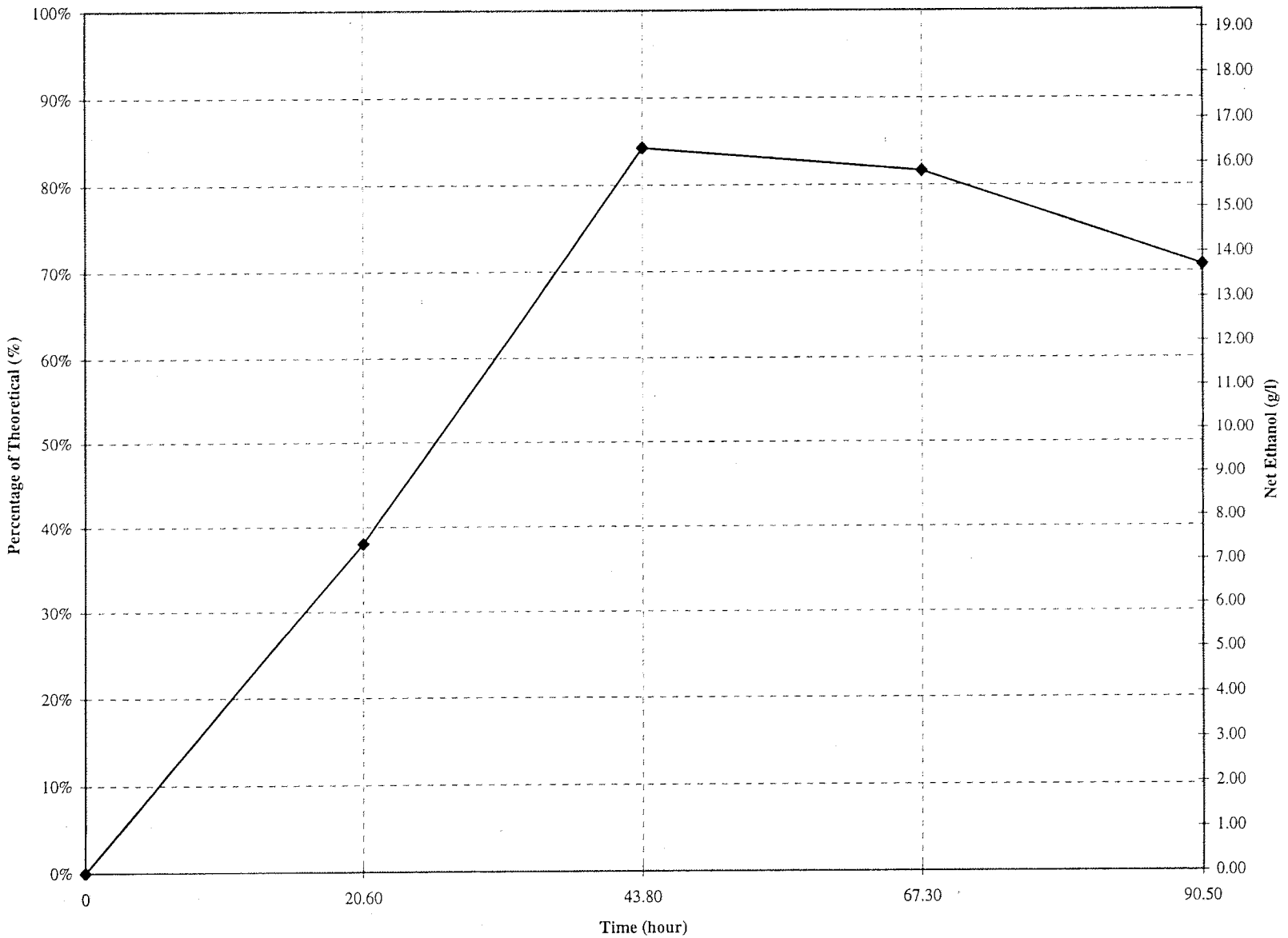

Figure 27 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 1, 80% hydrolysate.

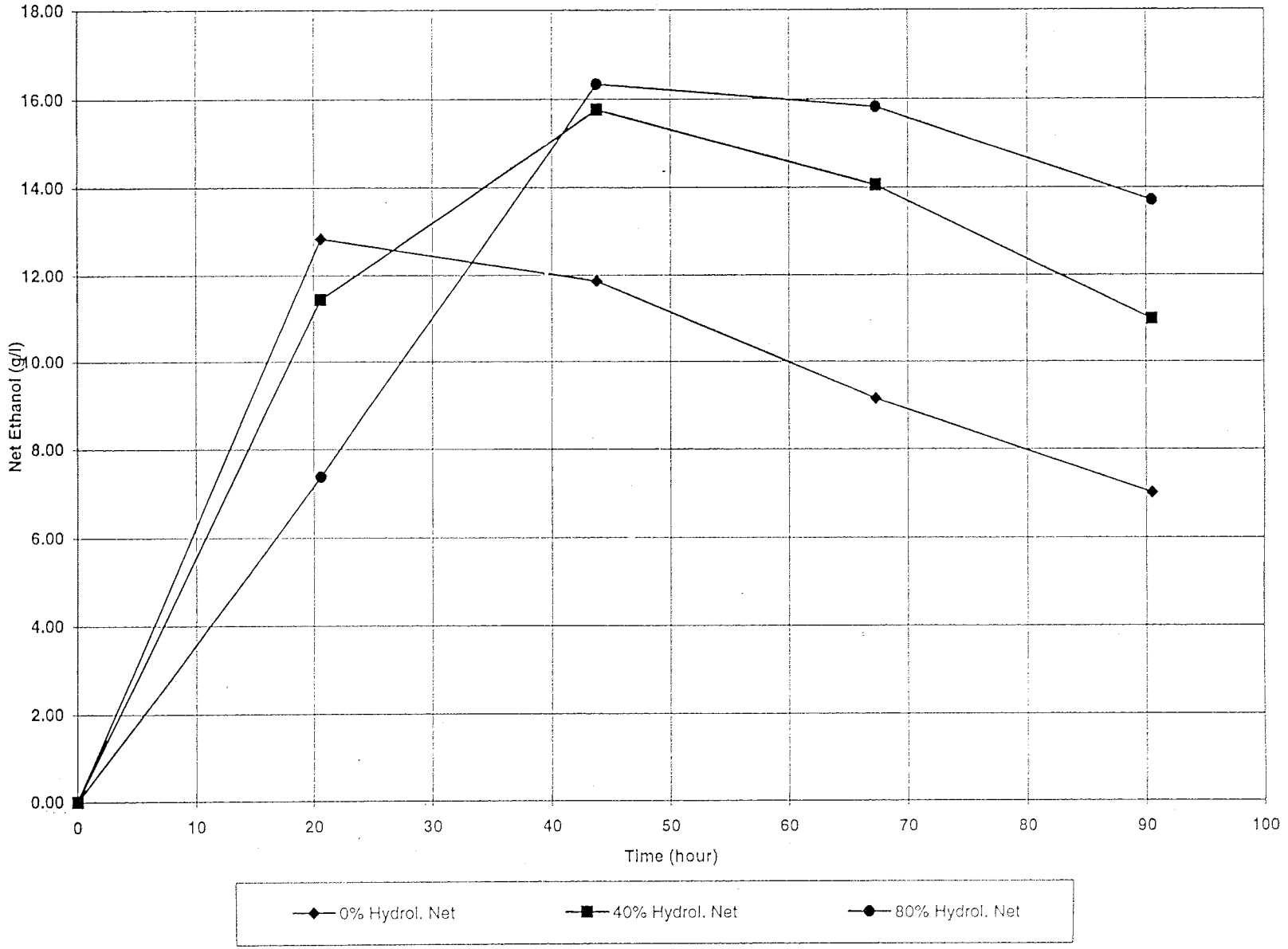

Figure 28 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 1, 0%-80% hydrolysate (average of 2 samples)

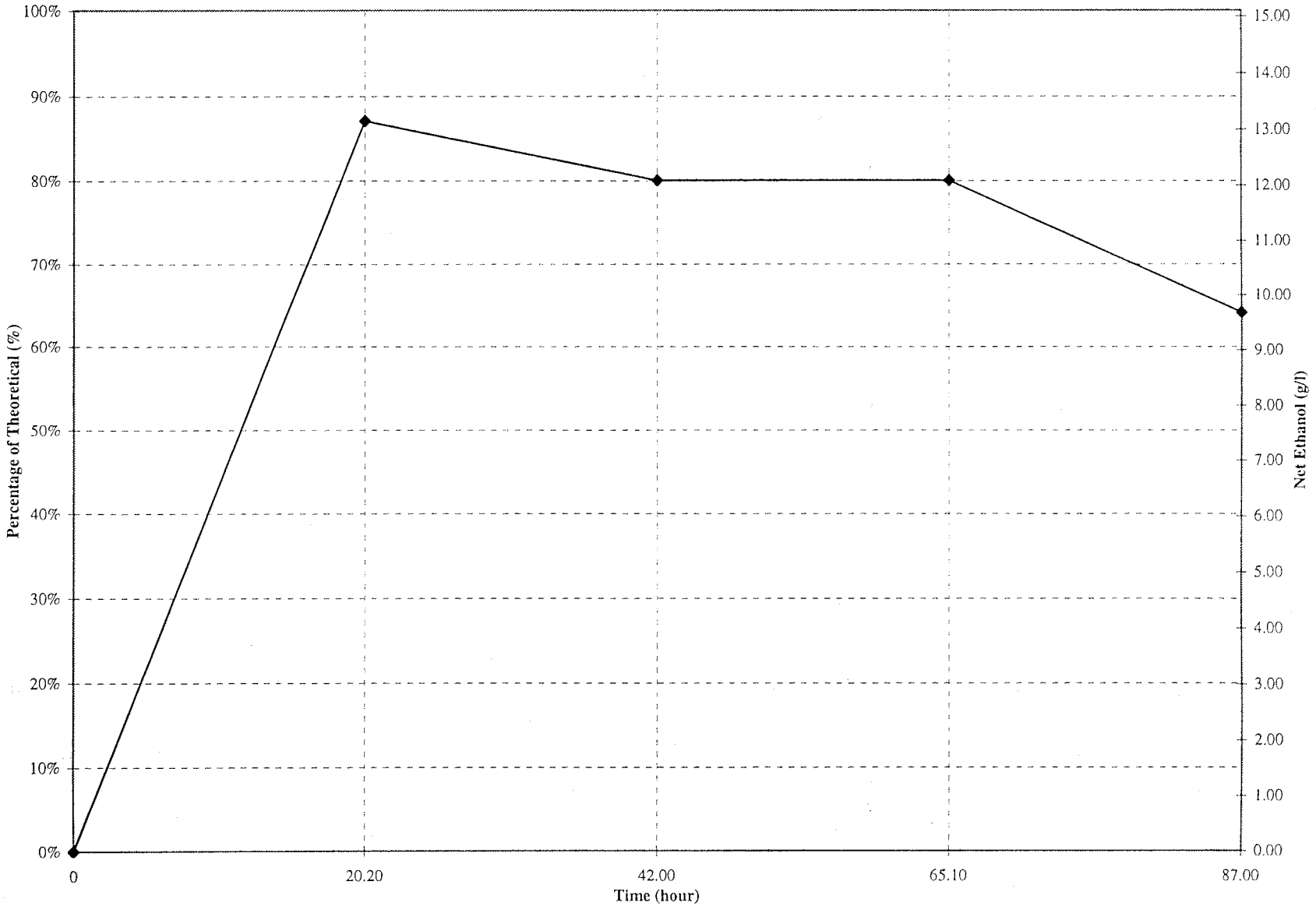

Figure 29 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 3, 0% hydrolysate

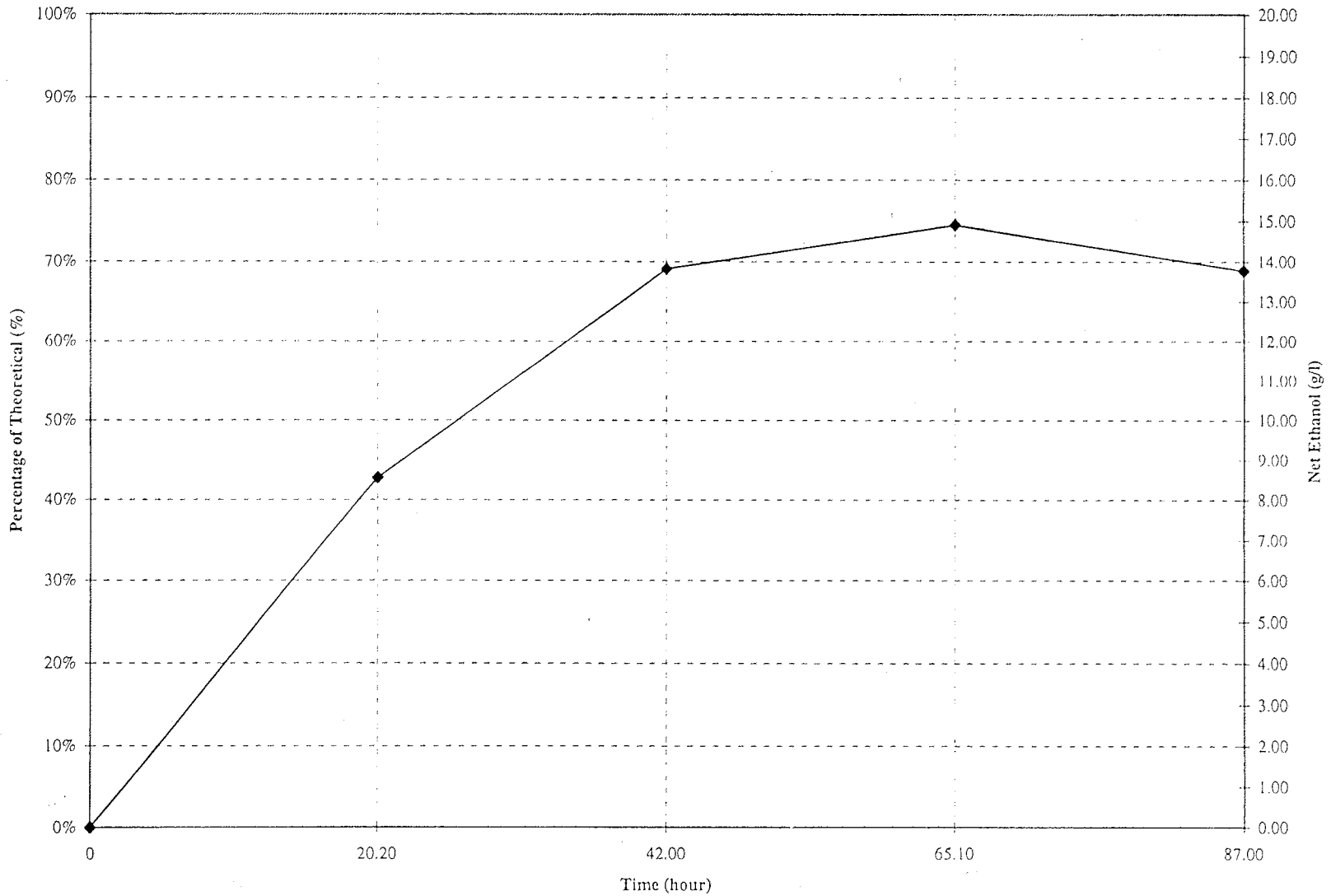

Figure 30 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 3, 40% hydrolysate

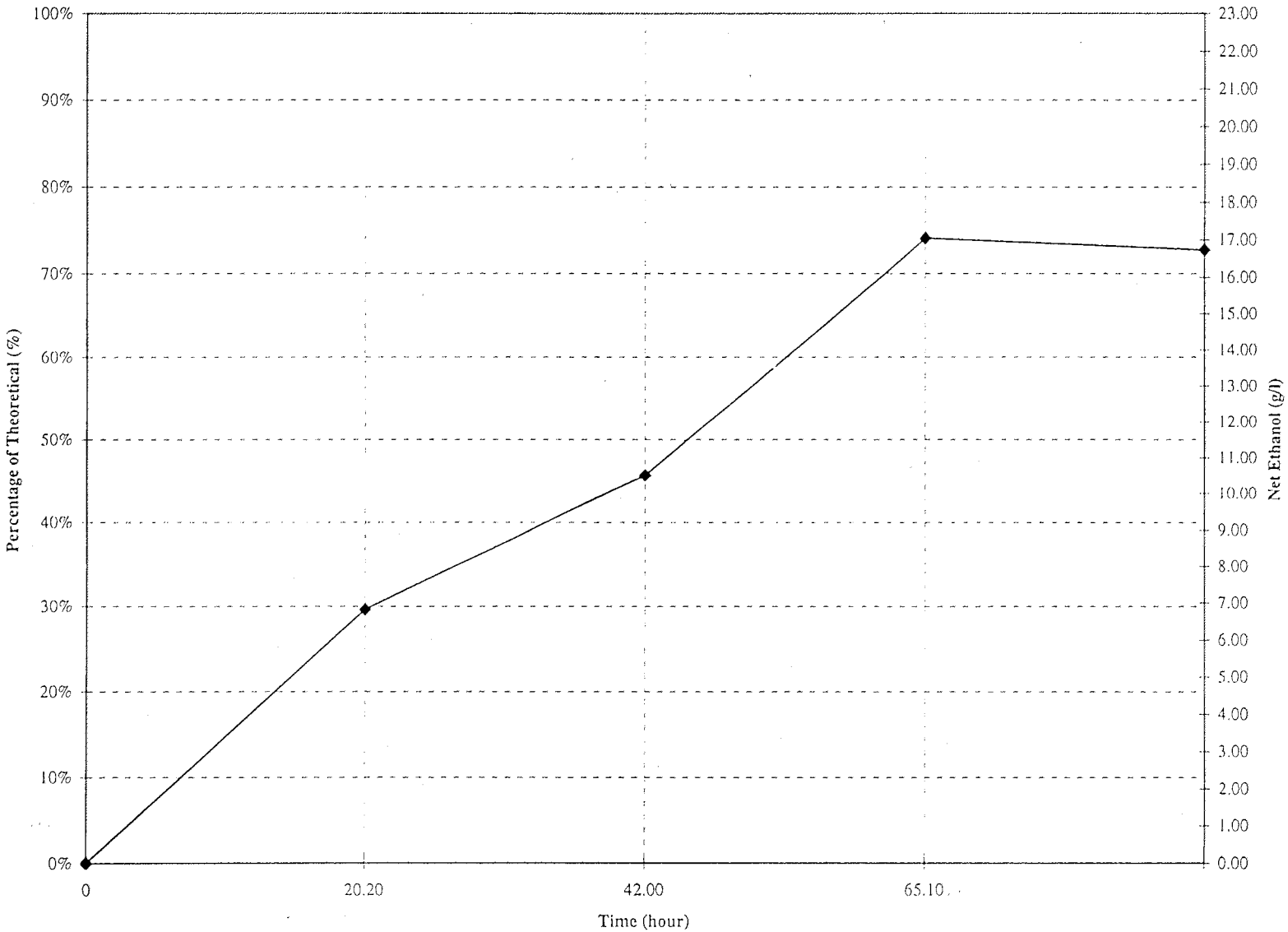

Figure 31 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 3, 80% hydrolysate

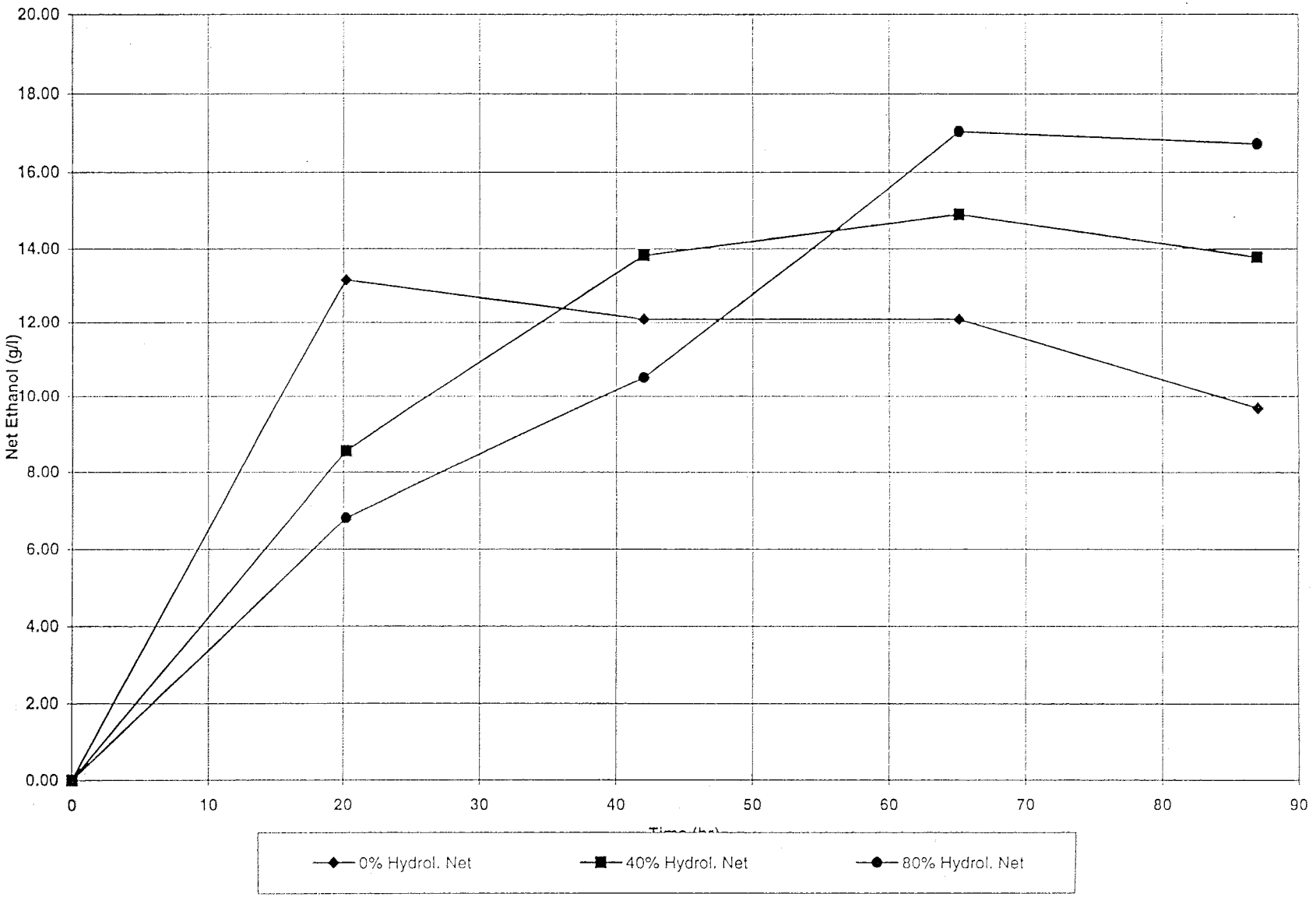

Figure 32 Net ethanol production in Pichia stipitis fermentation of hybrid poplar reaction 3, 0% -80% hydrolysate (average of 2 samples)

Table 24 Ethanol Yields in Pichia stipitis Fermentation of Switchgrass Reaction 3, 0%-90% Hydrolyzate

Hydrolyzate, %	0	40	80	90
Time Zero Concentration, g/l				
Glucose+Mannose	0.33	1.84	9.94	13.16
Xylose+Galactose	31.80	39.39	31.50	29.98
Cellobiose	0.00	0.28	3.05	3.40
Ethanol	1.16	1.16	1.16	1.16
Max. Net Ethanol Yield*, g/g	0.51	0.47	0.40	0.11
Max. Net Ethanol Concentration, g/l	16.47	19.59	17.74	5.00
Time to Max. Net Ethanol, hours	22.20	22.20	92.40	92.40
Average Net Ethanol Volume Productivity, g/l/hr	0.74	0.88	0.19	0.05
Maximum Theoretical Ethanol*, g/l	16.42	21.22	22.83	23.88
Experimental Ethanol Yield as Percentage of Theoretical Max.	100%	92%	78%	21%

* based on grams of total fermentable sugars present

Table 25 Ethanol Yields in Pichia stipitis Fermentation of Switchgrass Reaction 10, 0%-90% Hydrolyzate

Hydrolyzate, %	0	40	80	90
Time Zero Concentration, g/l				
Glucose+Mannose	0.33	3.05	7.86	9.34
Xylose+Galactose	31.80	34.63	35.40	33.24
Cellobiose	0.00	0.58	0.84	0.53
Ethanol	1.16	1.16	1.16	1.16
Max. Net Ethanol Yield*, g/g	0.51	0.44	0.36	0.30
Max. Net Ethanol Concentration, g/l	16.47	16.95	15.89	12.74
Time to Max. Net Ethanol, hours	22.20	45.30	68.80	68.80
Average Net Ethanol Volume Productivity, g/l/hr	0.74	0.37	0.23	0.19
Maximum Theoretical Ethanol*, g/l	16.42	19.57	22.54	22.03
Experimental Ethanol Yield as Percentage of Theoretical Max.	100%	87%	70%	58%

* based on grams of total fermentable sugars present

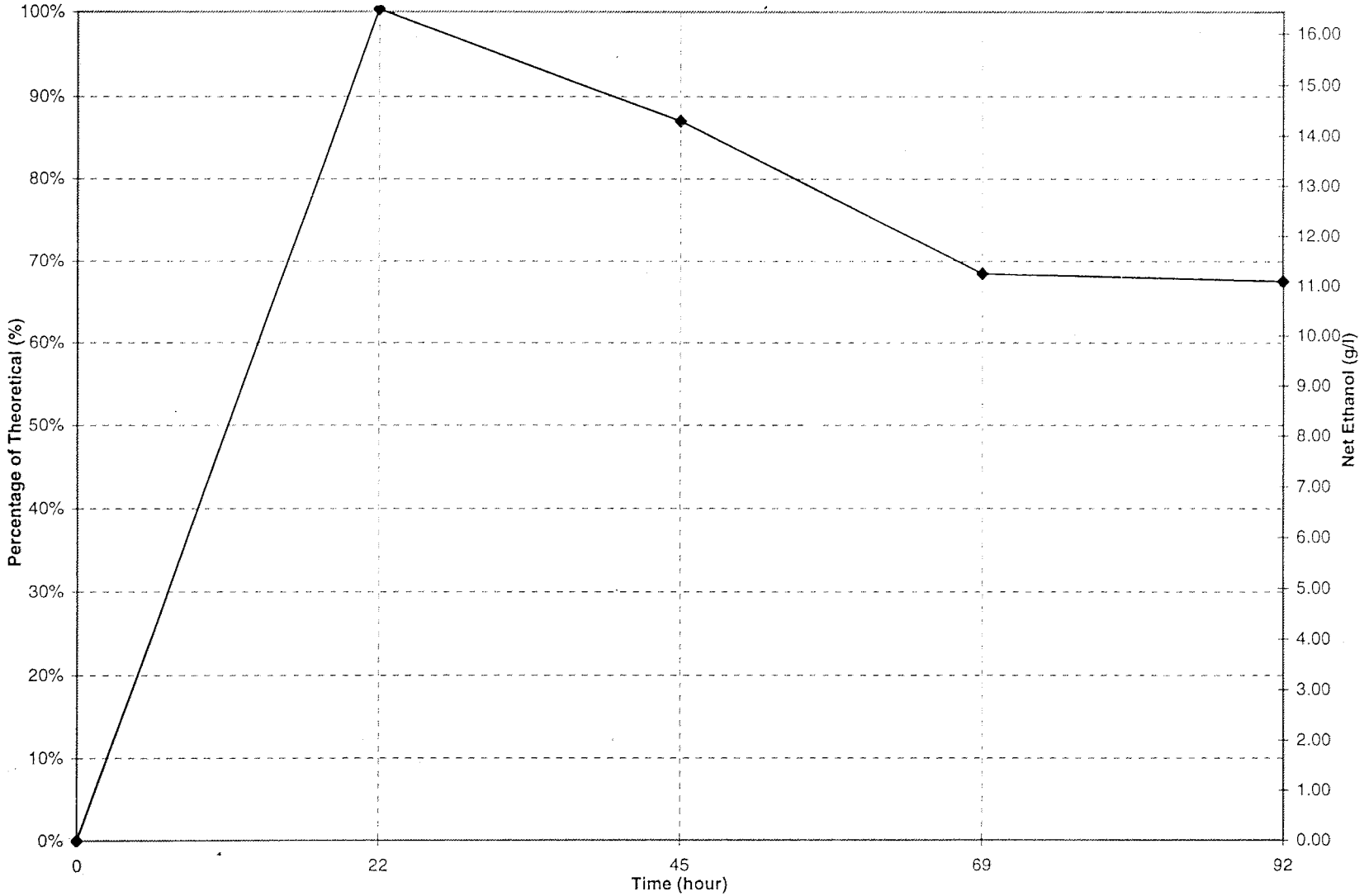

Figure 33 Net ethanol production in Pichia stipitis fermentation of switchgrass 0% hydrolysate

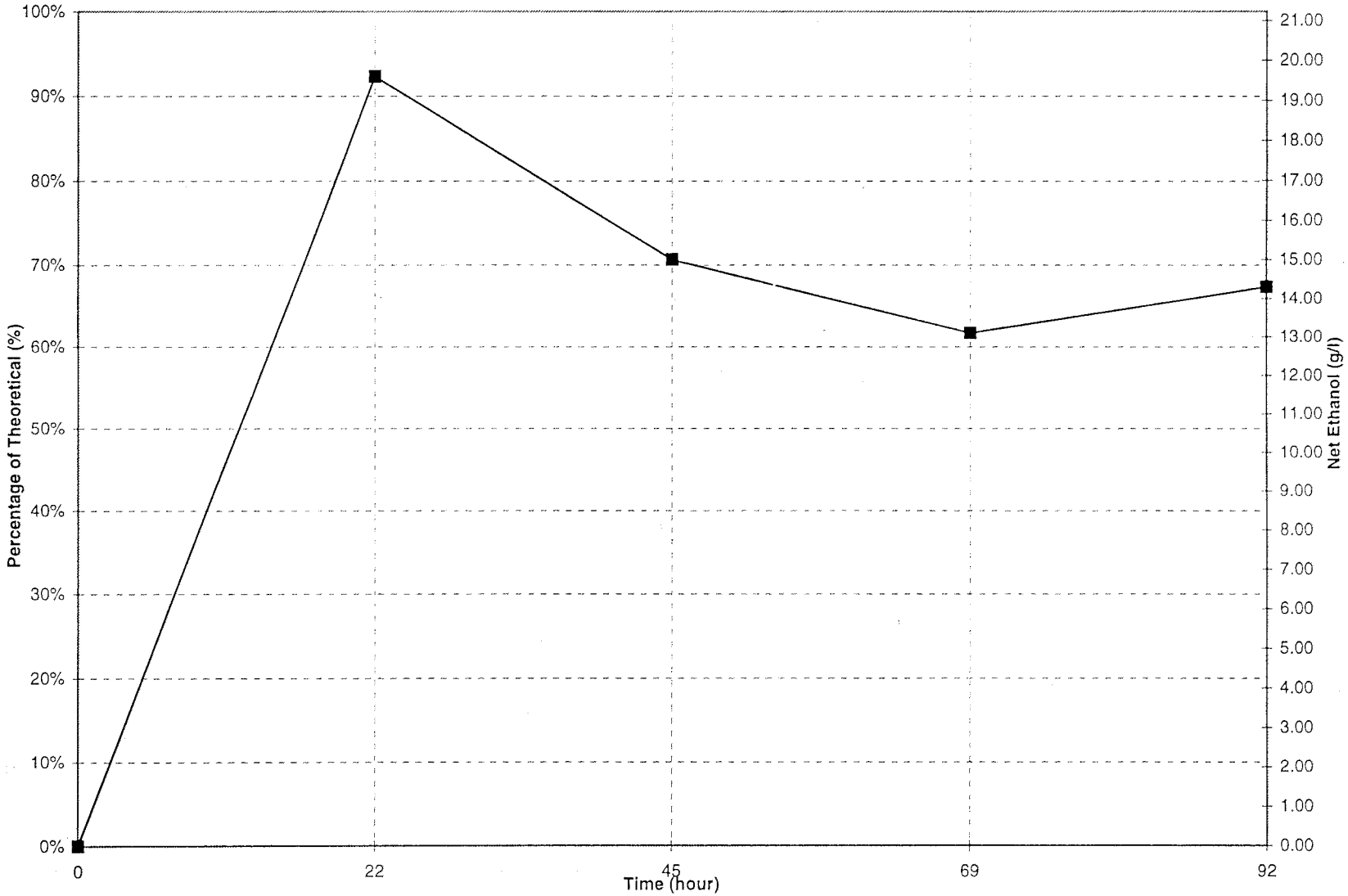

Figure 34 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 3, 40% hydrolysate

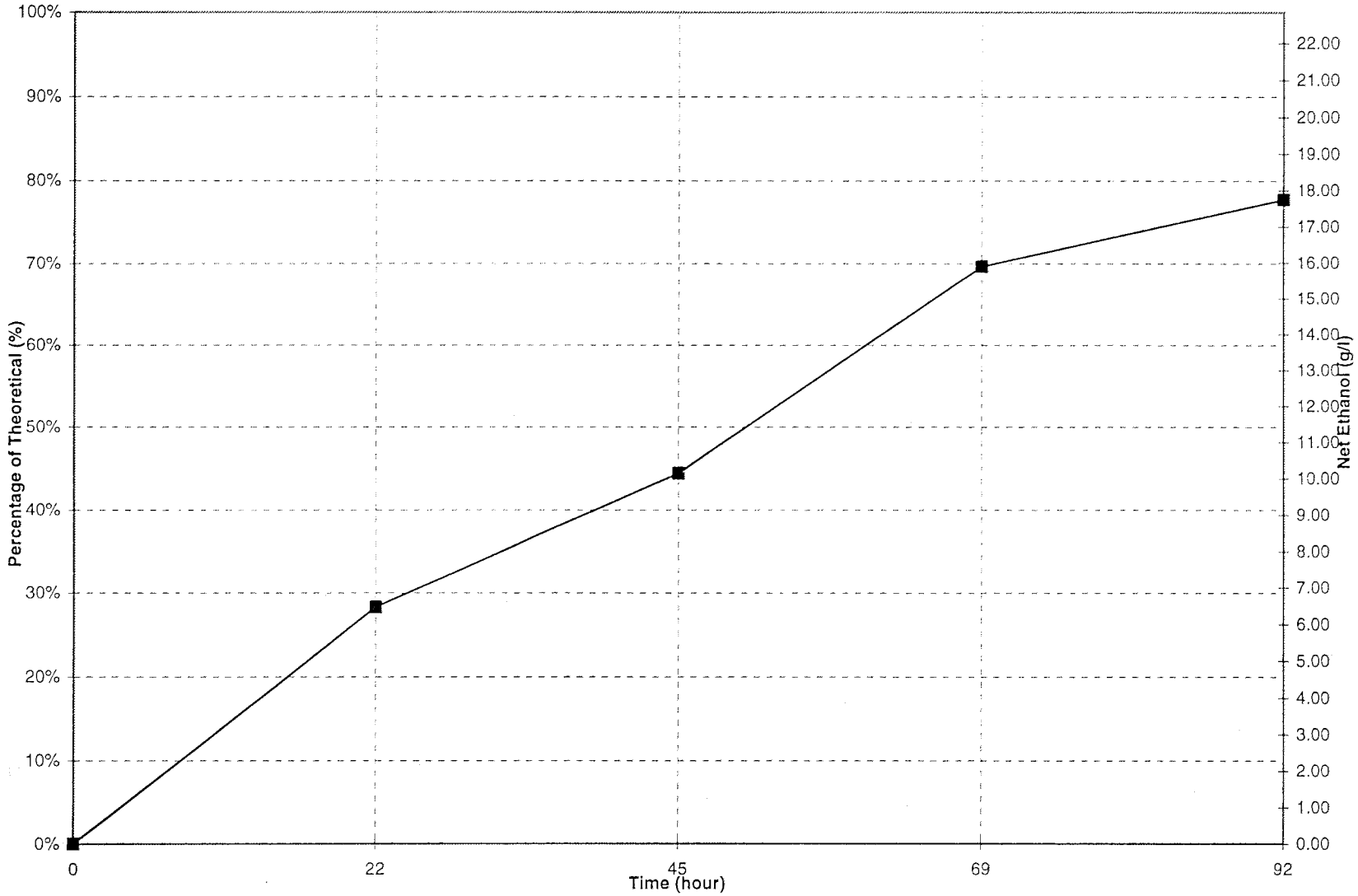

Figure 35 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 3, 80% hydrolysate

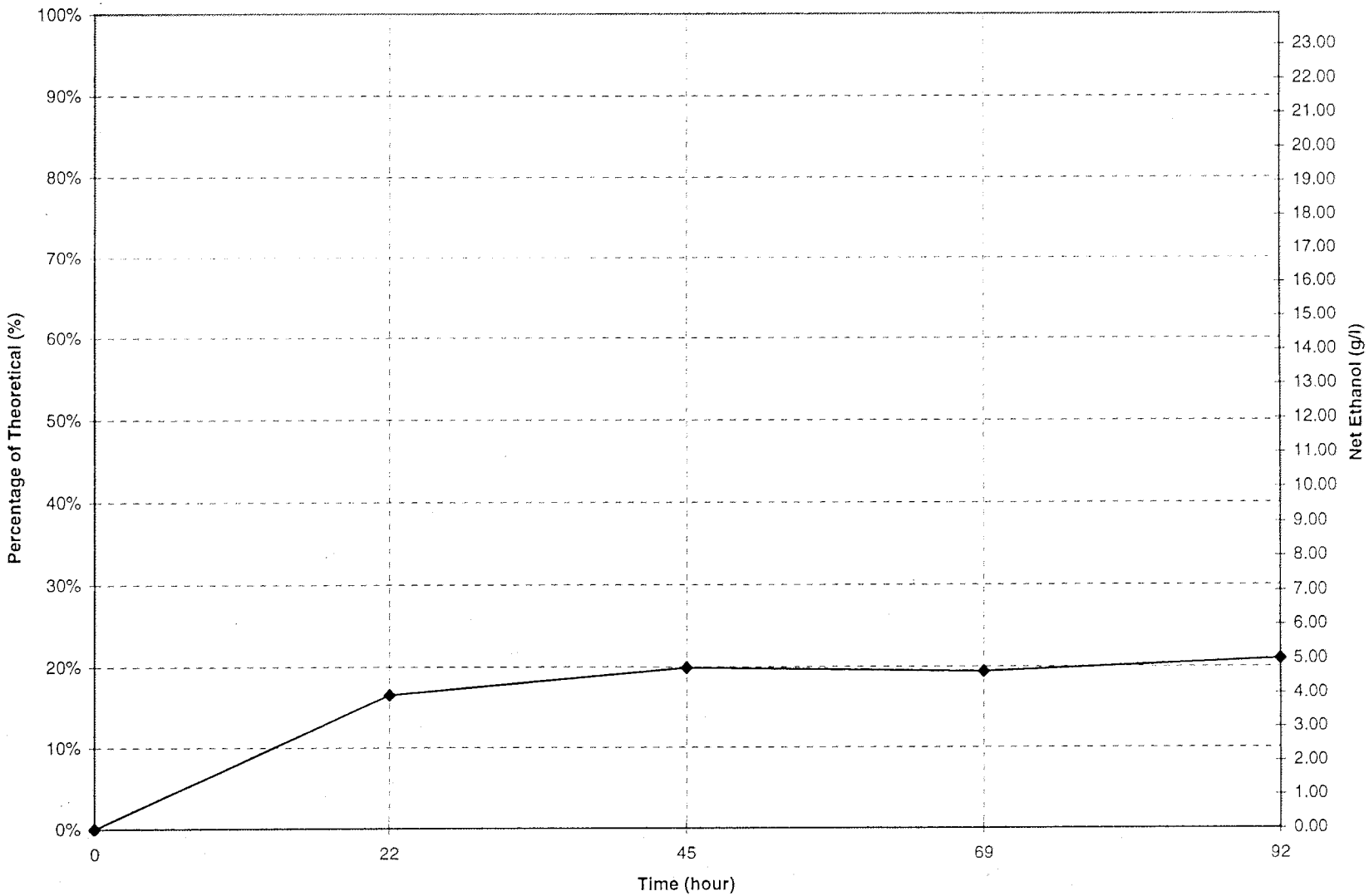

Figure 36 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 3, 90% hydrolysate

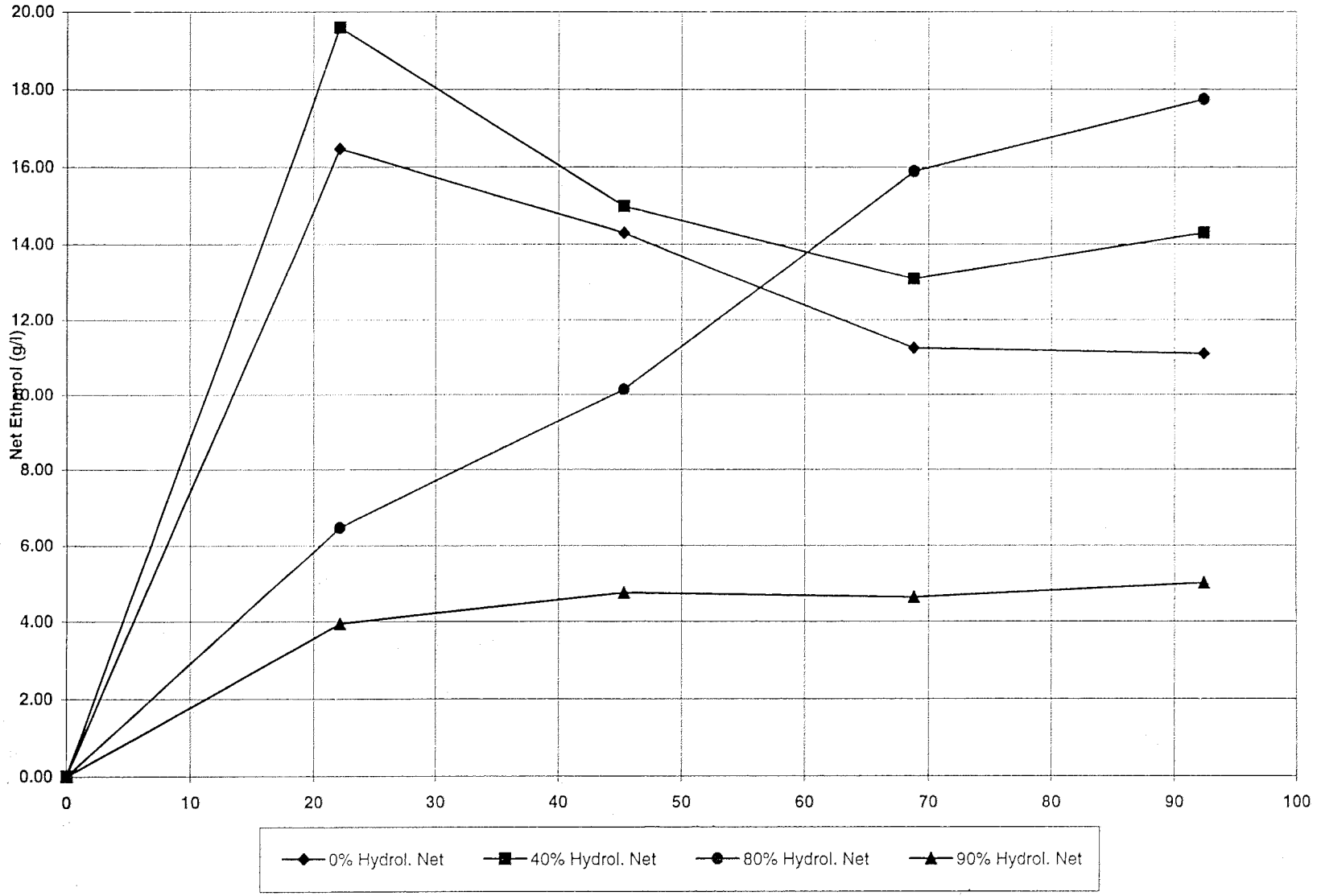

Figure 37 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 3, 0%-90% hydrolysate (average of 2 samples)

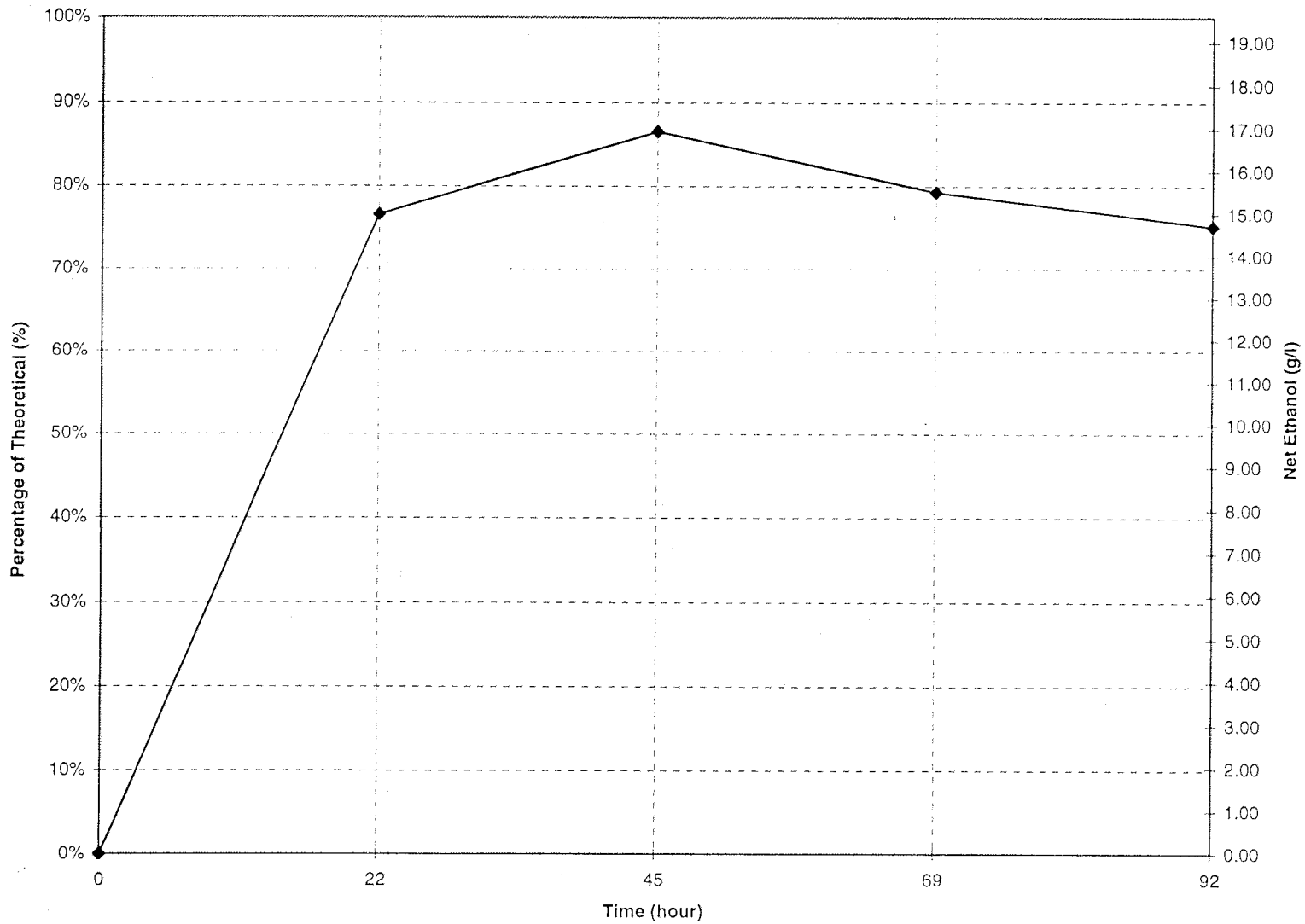

Figure 38 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 10, 40% hydrolysate

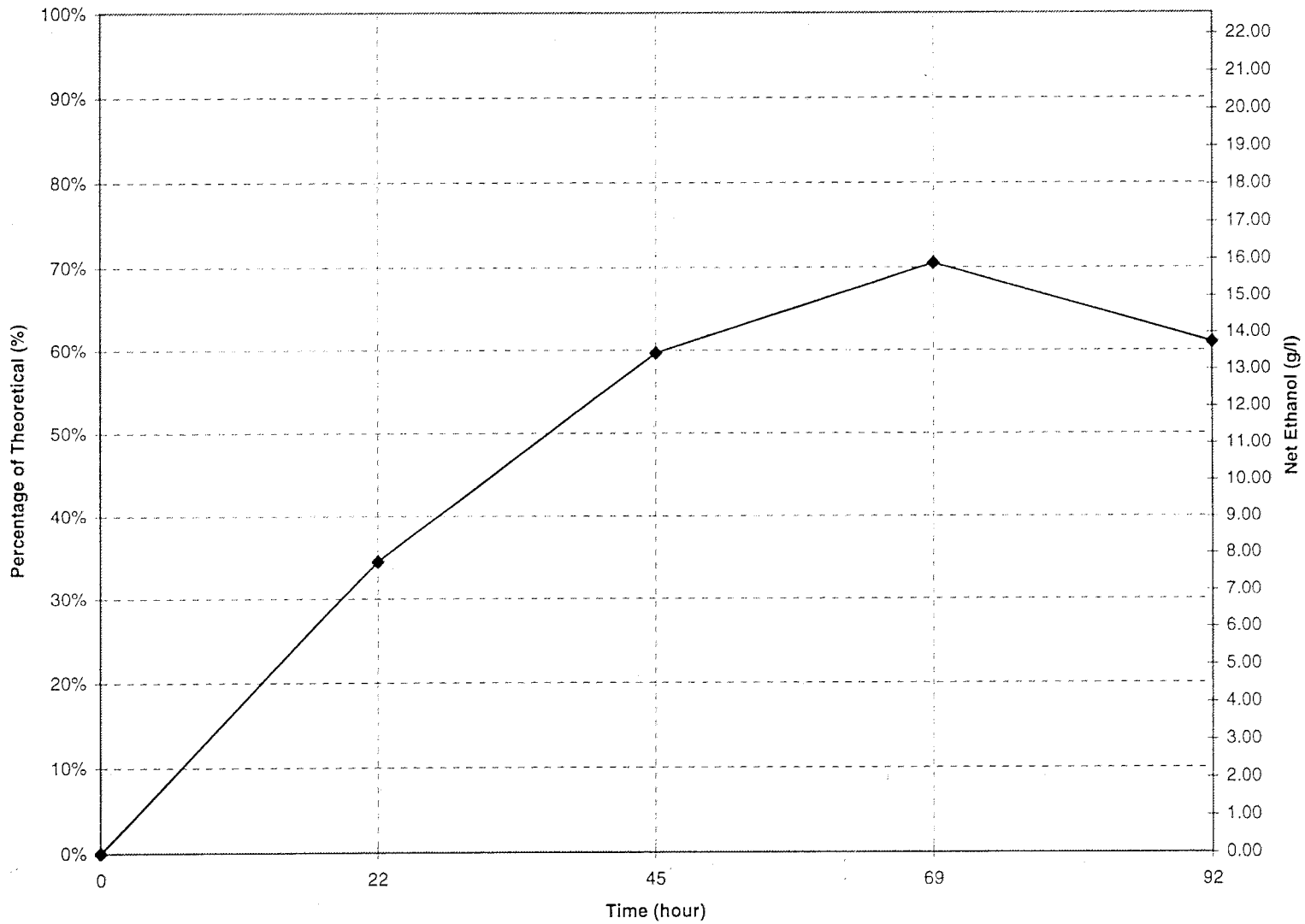

Figure 39 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 10, 80% hydrolysate

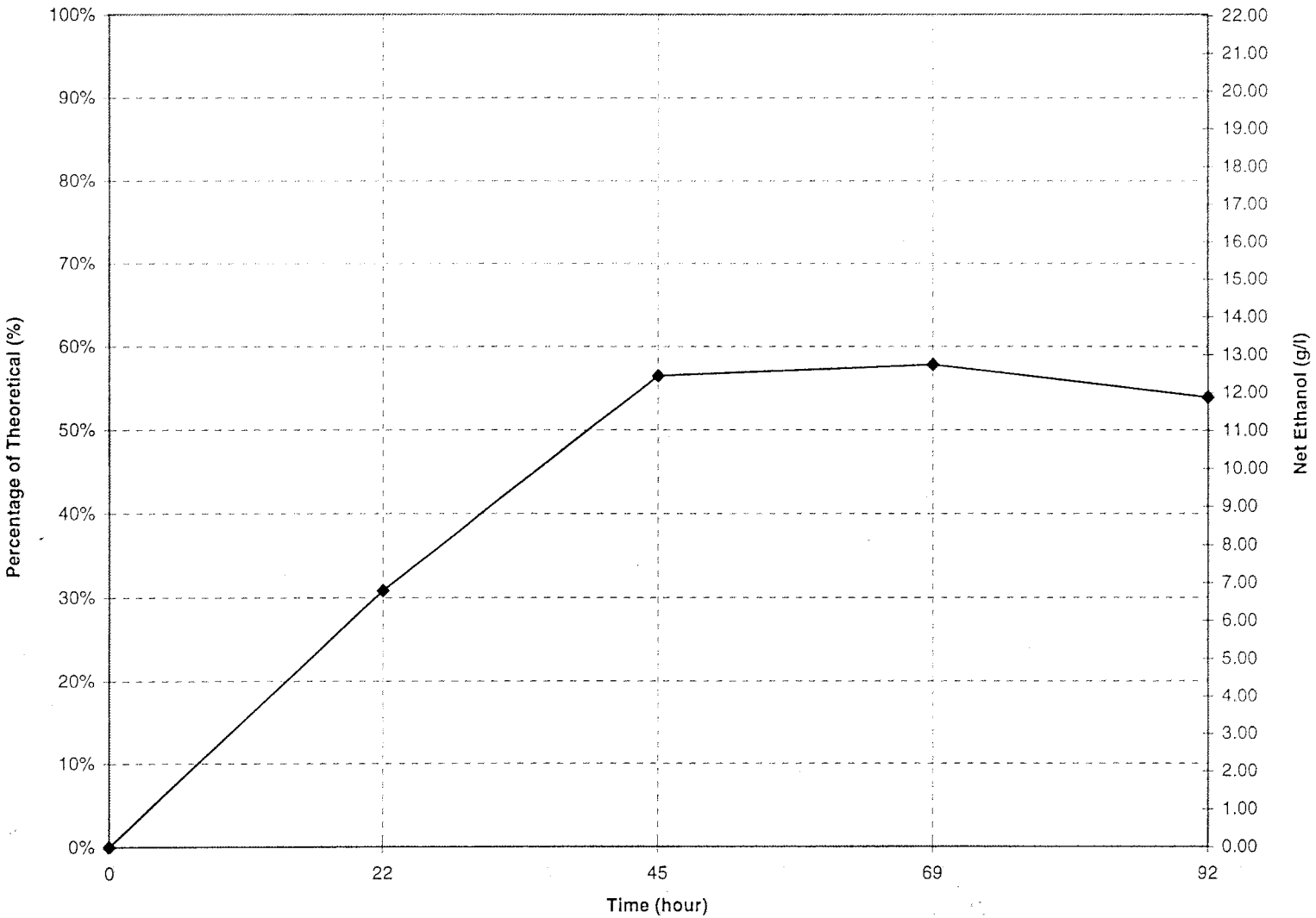

Figure 40 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 10, 90% hydrolysate

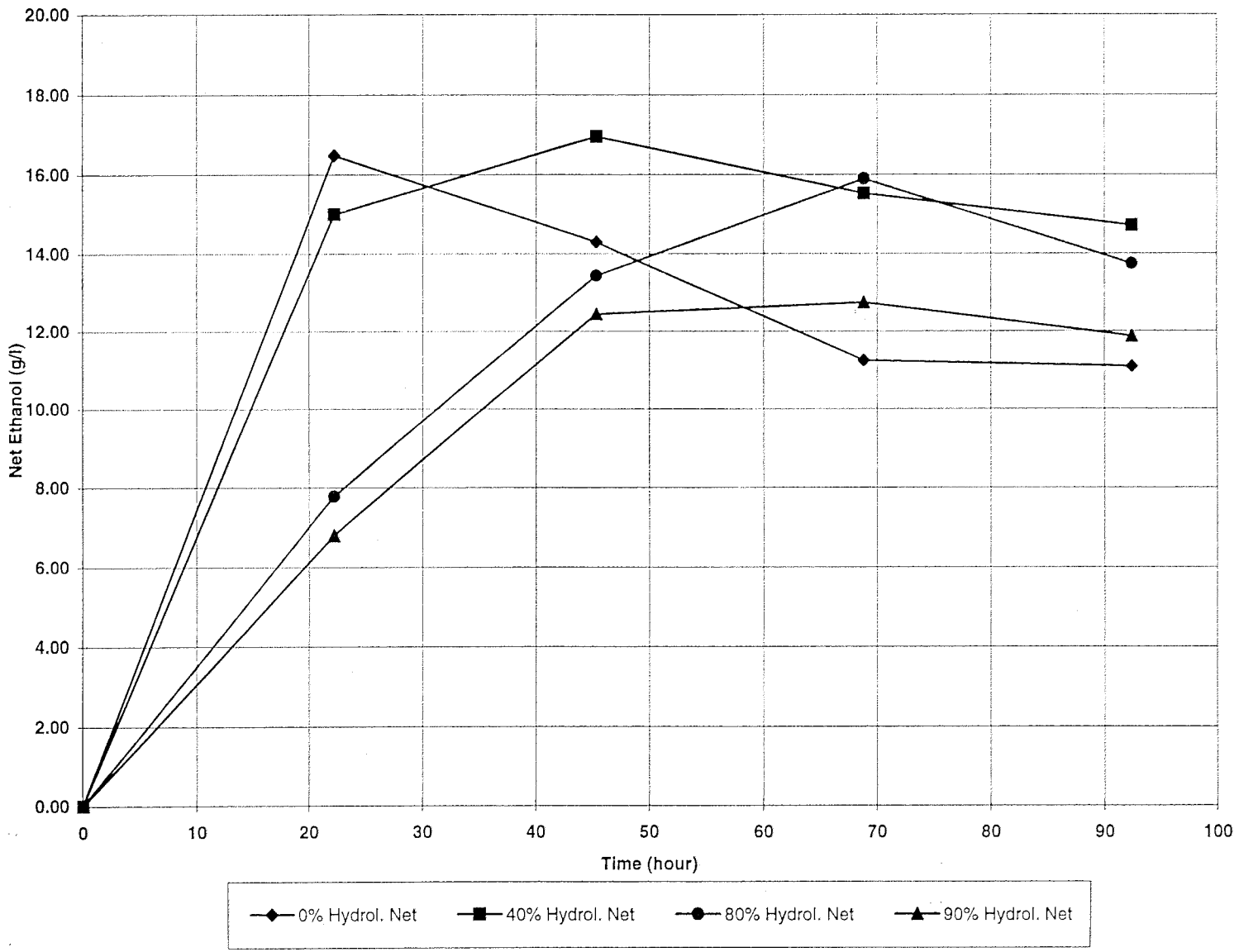

Figure 41 Net ethanol production in Pichia stipitis fermentation of switchgrass reaction 10, 0%-90% hydrolysate (average of 2 samples)

5.3 Products in *Pichia stipitis* Fermentation of Hybrid Poplar and switchgrass

HPLC analyses of *P. stipitis* fermentate of both hybrid poplar Reactions 1 and 3 hydrolysates were carried out using NREL's standard protocol for pretreatment liquor/xylose fermentation using *P. stipitis* NRRL Y11545. Samples were collected at nominal times of 00, 24, 48, 72 and 96 hours with actual times dependent upon time availability of researchers. Ethanol analyses in the fermentates were independently obtained by YSI. The production of net ethanol and other compounds in the fermentate were calculated. Results obtained on hybrid poplar Reaction 3 were sufficiently complete and critically reviewed in time for presentation of tables of data and overhead plots of data at the May 11-12, 1995 subcontractors meeting in Golden, Colorado.

The integrator program used with HPLC was revised in June, 1995, to provide information on minor peaks. In particular, three areas of the chromatograms of both hydrolysates as well as fermentates were examined in three zones. The major emphasis was placed on the zone just prior to the elution of glucose and at the retention time for cellobiose and other possible oligosaccharides. The literature on this subject was given particular attention. An unknown peak, eluting at a retention time of 53.5 minutes was found and its characterization initiated. The presence of other unknown but minor peaks was investigated. HPLC columns used were both BioRad HPX-87P and HPX-87H.

The minor as well as major products in *P. stipitis* fermentations of hybrid poplar and switchgrass are shown in Tables 26-38 and Figures 42-54.

5.3.1 Products of Hybrid Poplar Fermentation with Pichia stipitis

The products of *P. stipitis* fermentation of hybrid poplar are given in Tables 26-31 and Figures 42-47.

5.3.1.1 Hybrid Poplar Reaction 1.

The products of hybrid poplar Reaction 1, 0% hydrolysate are given in **Table 26** and **Figure 42**.
The products of 40% hydrolysate are shown in **Table 27** and **Figure 43**. The products of 80% hydrolysate are given in **Table 28** and **Figure 44**.

No significant minor products were found in hybrid poplar Reaction 1.

Table 26 Products in Pichia stipitis Fermentation of Hybrid Poplar Reaction 1, 0% hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.52	1.54		0.00	0.00	24.14	0.00	0.00	5.59
		2	1.48	1.47		0.00	0.00	24.27	0.17	0.00	
24	20.6	1	15.25	14.61		0.00	0.00	0.54	0.33	0.23	8.29
		2	15.15	14.05		0.00	0.00	0.00	0.38	0.00	
48	43.8	1	10.85	12.94		0.00	0.00	0.00	0.31	0.20	11.93
		2	13.00	13.78		0.00	0.00	0.00	0.35	0.21	
72	67.3	1	12.10	10.41		0.00	0.00	0.00	0.26	0.14	10.75
		2	13.40	10.87		0.00	0.00	0.00	0.29	0.10	
96	90.5	1	10.01	8.73		0.00	0.00	0.00	0.32	0.22	11.48
		2	10.70	8.28		0.00	0.00	0.26	0.34	0.21	

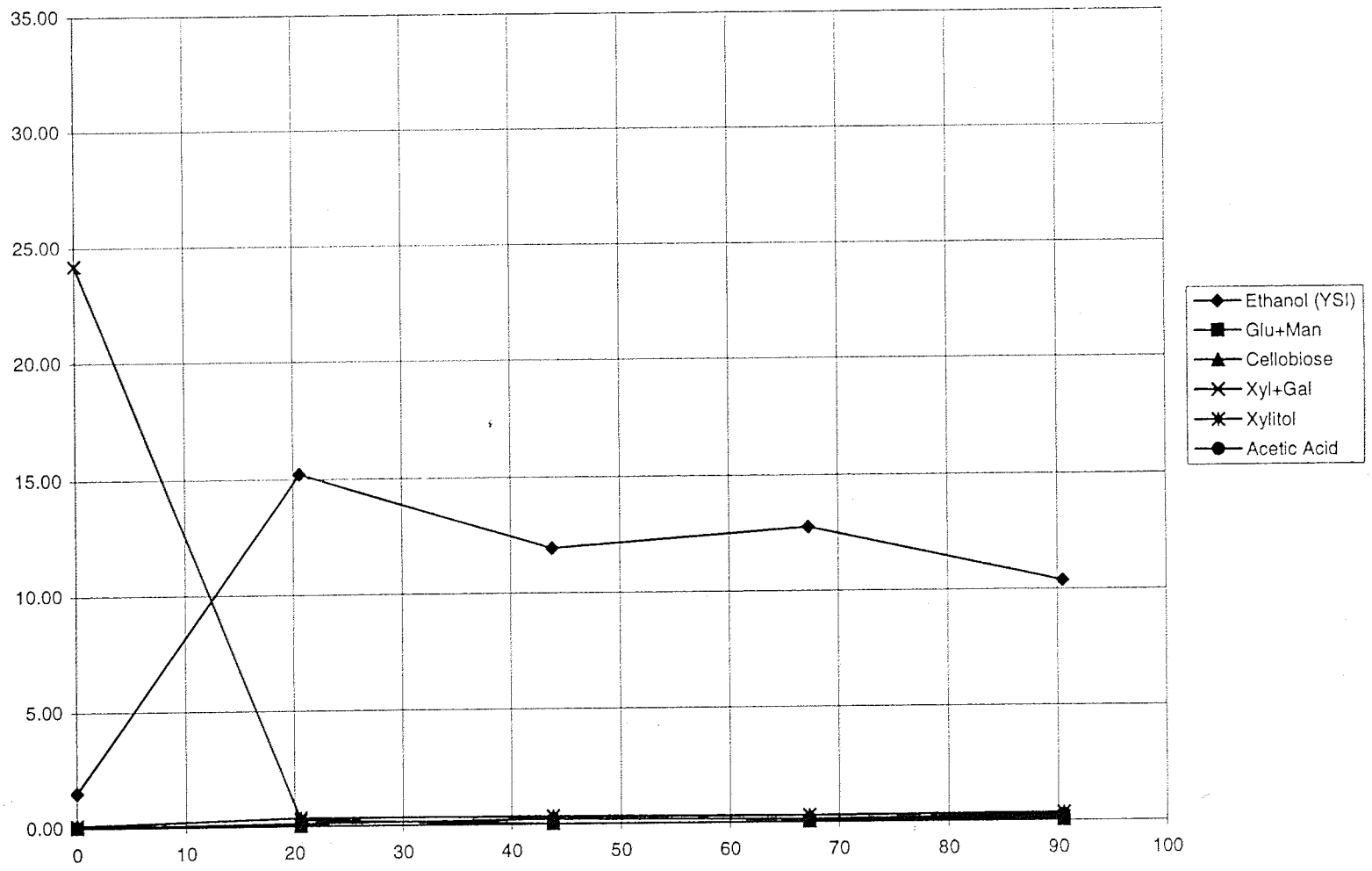

Figure 42 Products in Pichia stipitis fermentation of hybrid poplar reaction 1, 0% hydrolysate

Table 27 Products in Pichia stipitis Fermentation of Hybrid Poplar Reaction 1, 40% hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.51	1.54		1.20	0.69	30.03	0.17	0.96	
		2	1.49	1.44		1.10	0.66	29.97	0.17	0.84	
24	20.6	1	13.10	13.09		0.00	0.15	9.27	0.26	0.79	5.39
		2	13.35	12.78		0.00	0.16	9.17	0.21	0.69	
48	43.8	1	16.70	17.13		0.00	0.40	0.63	0.72	0.52	9.27
		2	16.90	17.39		0.00	0.45	0.62	0.76	0.55	
72	67.3	1	19.00	15.65		0.00	0.51	0.35	0.58	0.08	6.19
		2	18.70	15.46		0.00	0.48	0.35	0.27	0.05	
96	90.5	1	15.65	12.35		0.00	0.66	0.70	0.35	0.11	7.78
		2	15.75	12.61		0.00	0.90	0.71	0.81	0.11	

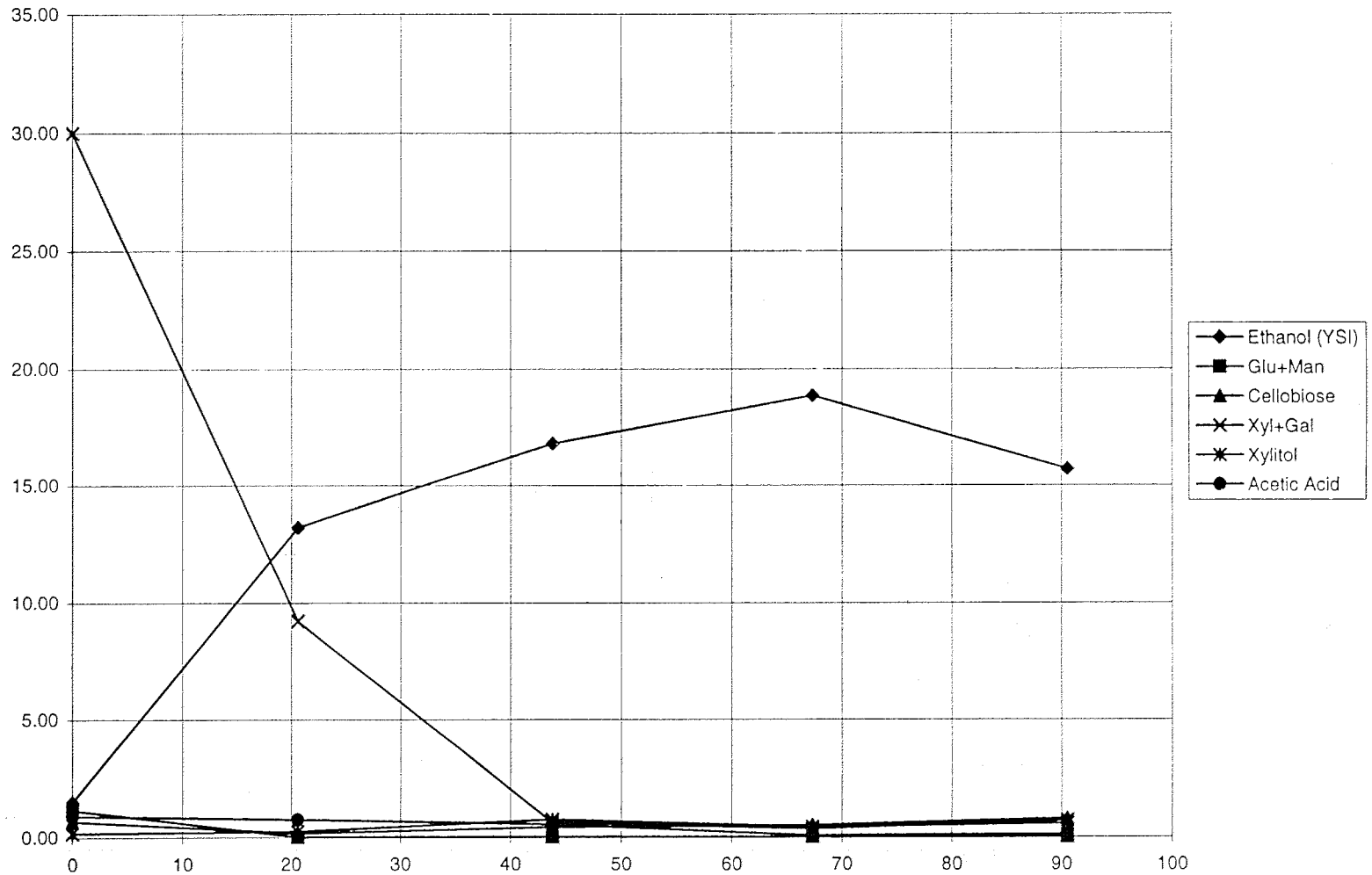

Figure 43 Products in Pichia stipitis fermentation of hybrid poplar reaction 1, 40% hydrolysate

Table 28 Products in Pichia stipitis Fermentation of Hybrid Poplar Reaction 1, 80% hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.72	2.01		2.34	1.03	34.11	0.20	1.59	
		2	1.60	2.15		2.69	0.50	35.08	0.18	1.57	
24	20.6	1	9.43	9.02		0.00	0.69	19.92	1.20	1.38	3.76
		2	9.45	8.73		0.00	0.75	20.47	1.19	1.42	
48	43.8	1	17.60	17.00		0.00	0.74	5.99	1.85	1.85	7.72
		2	17.60	18.67		0.00	0.77	6.33	1.82	1.66	
72	67.3	1	20.50	17.78		0.00	0.76	0.23	1.28	1.10	4.58
		2	21.90	16.86		0.00	1.41	1.75	1.94	1.60	
96	90.5	1	19.30	15.07		0.00	1.21	0.71	1.14	0.27	6.94
		2	17.35	15.35		0.00	1.33	1.02	1.71	0.75	

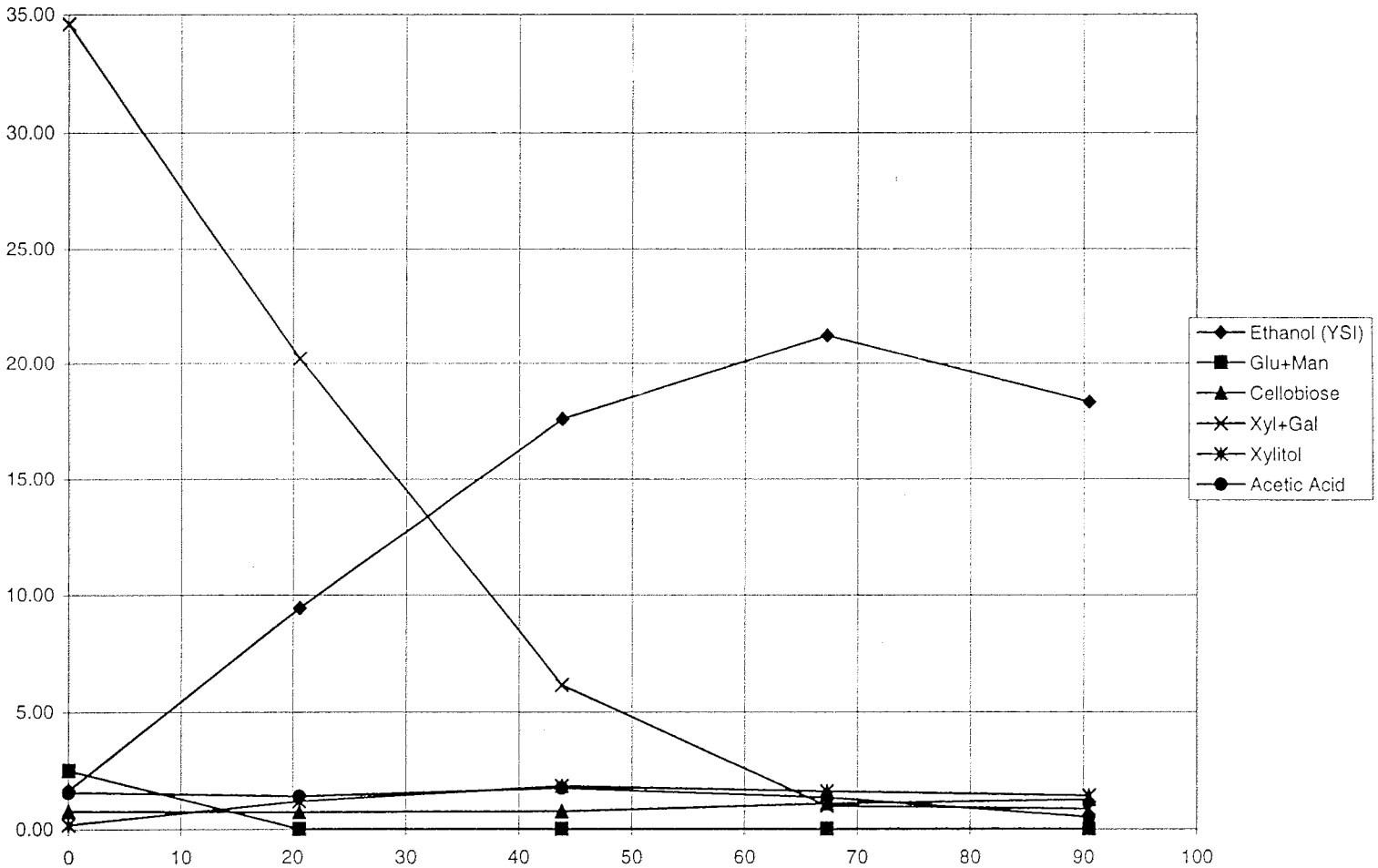

Figure 44 Products in Pichia stipitis fermentation of hybrid poplar reaction 1, 80% hydrolysate

5.3.1.2 Hybrid Poplar Reaction 3

The products of hybrid poplar Reaction 3, 0% hydrolysate, are those given in **Table 29** and **Figure 45.** The products of 40% hydrolysate are given in **Table 30** and **Figure 46**. Those of 80% hydrolysate are given in **Table 31** and **Figure 47**. Those of 90% hydrolysate are shown in **Table 32** and **Figure 48**.

Products in *P. stipitis* fermentation of H.P. Reaction 3, 80% hydrolysate Figure 47 shows a slow utilization of xylose. Minor products, particularly acetic acid are enhanced. Note that the hexoses i.e. glucose plus mannose are utilized preferentially with concentrations falling to near zero by 20 hours.

Table 29 Pichia stipitis Fermentation of Hybrid Poplar Reaction 3, 0% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	0.62			0.25	0.37	28.62	0.00	0.00	5.01
		2	0.62			0.42	0.39	28.96	0.00	0.12	
24	20.2	1	13.58			0.00	0.01	0.68	0.41	0.16	8.72
		2	13.82			0.00	0.00	0.99	0.45	0.18	
42	42	1	12.77			0.00	0.00	0.03	0.28	0.17	9.17
		2	12.50			0.00	0.00	0.05	0.29	0.29	
65	65.1	1	12.91			0.00	0.01	0.00	0.29	0.20	
		2	12.36			0.17	0.00	0.00	0.32	0.10	
87	87	1	10.17			0.00	0.00	0.33	0.37	0.00	9.47
		2	10.29			0.00	0.00	0.45	0.38	0.20	

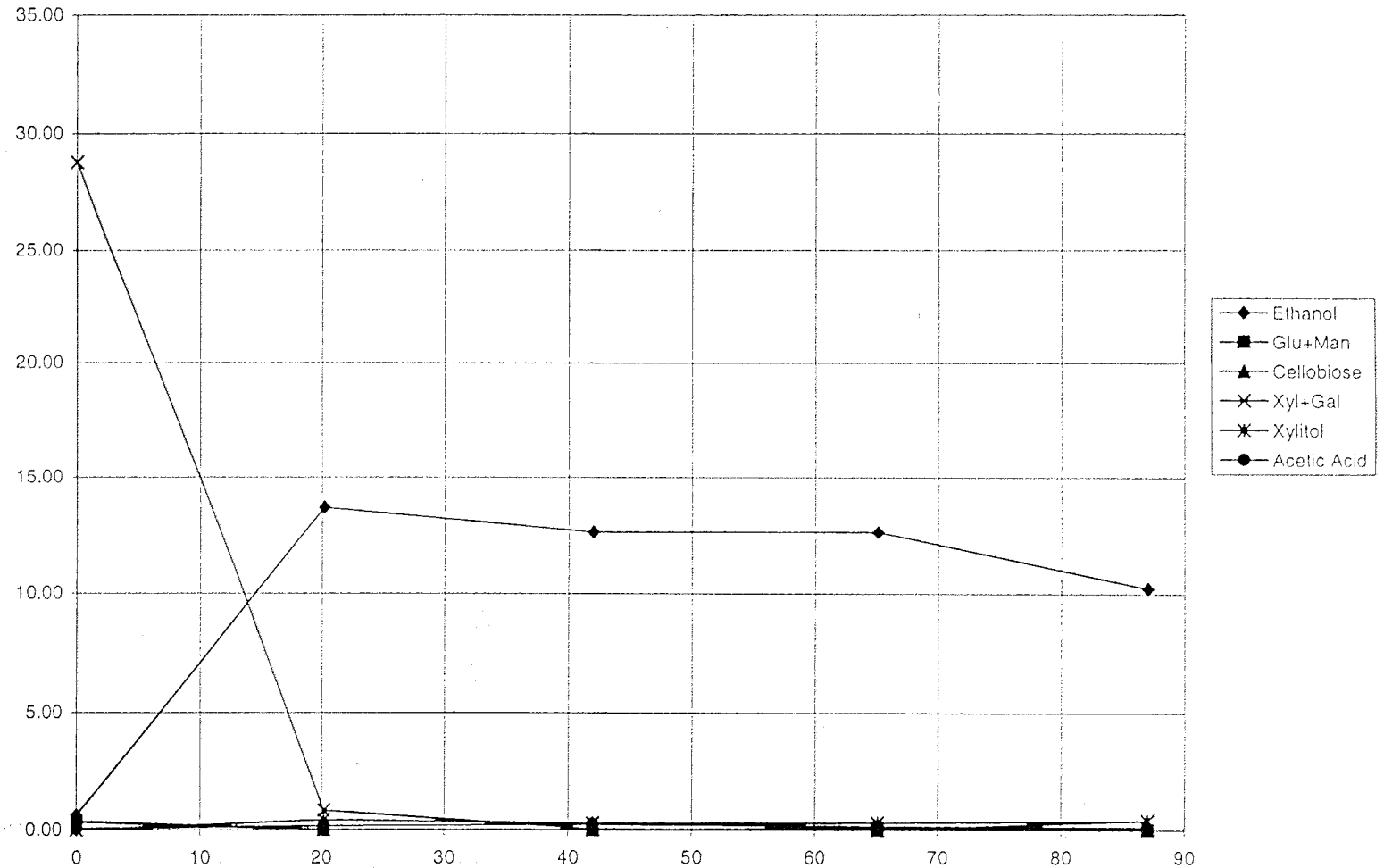

Figure 45 Products in Pichia stipitis fermentation of hybrid poplar reaction 3, 0% hydrolysate

Table 30 Products in Pichia stipitis Fermentation of Hybrid Poplar Reaction 3, 40% hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	0.6			3.2	1.7	32.29	0.04	1.26	
		2	0.54			4.39	1.19	36.11	0.05	1.60	
24	20.2	1	9.35			0.00	0.19	11.36	0.46	1.27	
		2	8.85			0.00	0.19	11.99	0.45	1.24	
42	42	1	14.19			0.00	0.34	0.88	0.60	0.90	6.91
		2	14.55			0.00	0.33	0.47	0.62	0.47	
65	65.1	1	15.68			0.00	0.60	0.58	0.67	0.37	
		2	15.22			0.00	0.34	0.12	0.49	0.00	
87	87	1	13.74			0.00	0.39	0.50	0.71	0.00	6.71
		2	14.90			0.00	0.41	0.35	0.72	0.00	

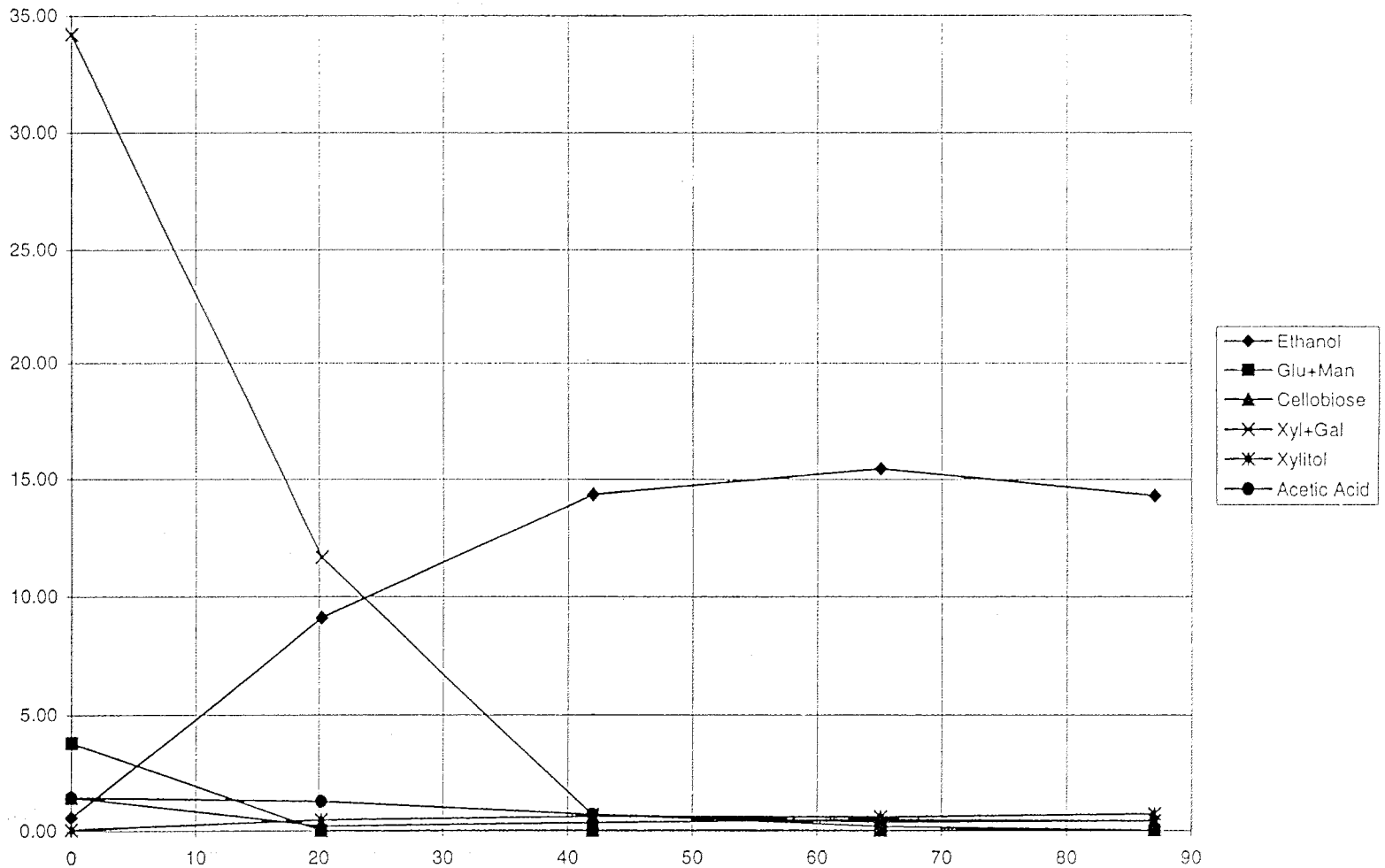

Figure 46 Products in Pichia stipitis fermentation of hybrid poplar reaction 3,40% hydrolysate

Table 31 Products in Pichia stipitis Fermentation of Hybrid Poplar Reaction 3, 80% Hydrolyzate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol HPLC	Ethanol YSI	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	0.42			7.30	1.44	35.38	0.05	2.64	
		2	0.52			7.88	1.44	35.71	0.03	2.88	
24	20.2	1	7.36			0.45	0.34	19.71	0.14	2.13	
		2	7.37			0.00	0.39	22.98	0.11	2.37	
42	42	1	10.01			0.00	0.35	5.90	0.74	1.33	6.26
		2	12.10			0.00	0.43	9.32	0.52	1.99	
65	65.1	1	17.35			0.00	1.04	0.95	0.77	1.72	
		2	17.82			0.00	0.89	1.11	0.82	1.89	
87	87	1	17.43			0.00	0.97	0.49	0.86	0.84	7.33
		2	17.12			0.00	0.96	0.51	0.85	0.83	

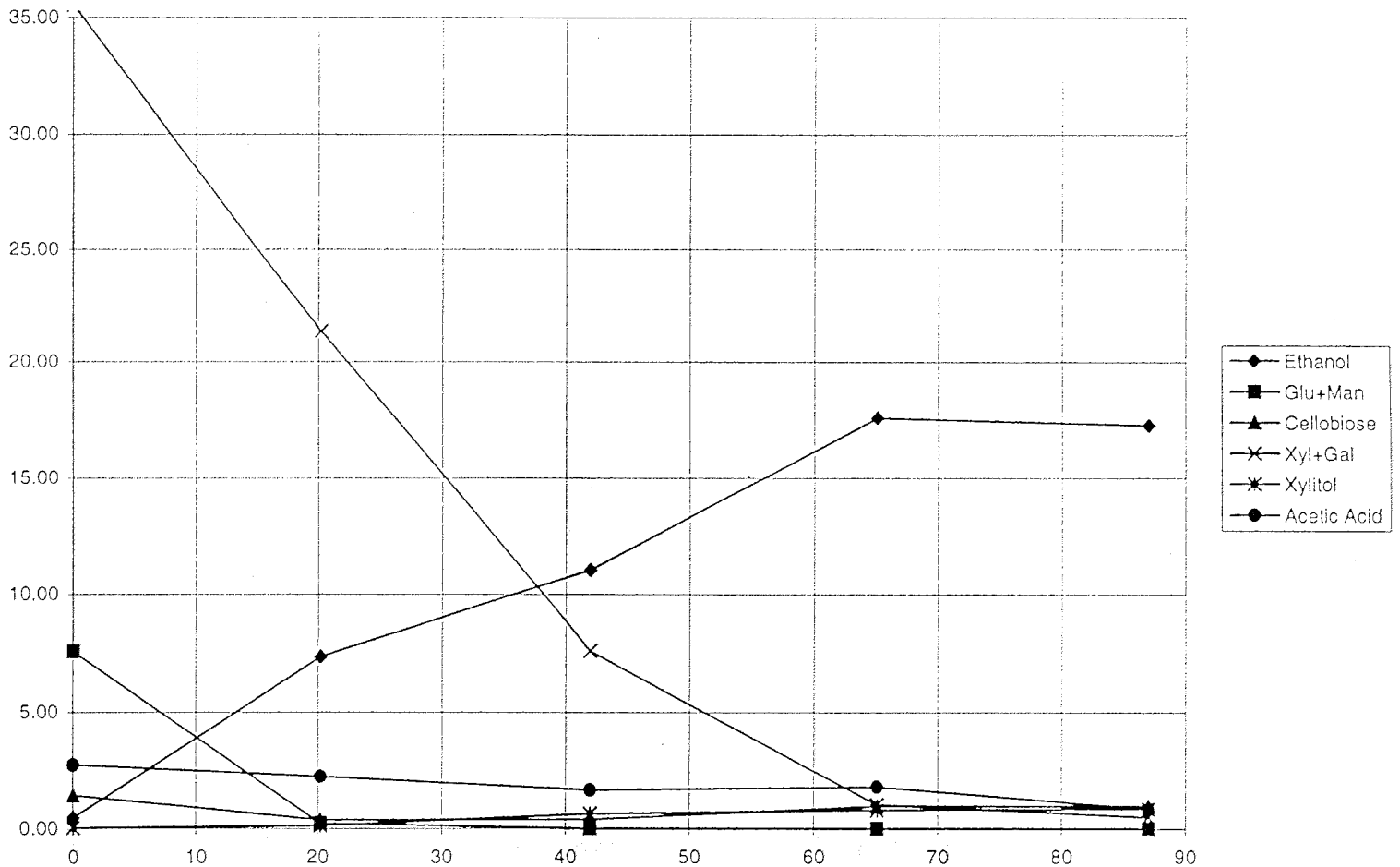

Figure 47 Products in Pichia stipitis fermentation of hybrid poplar reaction 3, 80% hydrolyzate

Table 32 Products in Pichia stipitis Fermentation of Switchgrass Reaction 3 and Reaction 10, 0% Hydrolysate

			Concentration (g/l)								
Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.21	1.22	0.33	0.00	0.00	31.52	0.05	0.00	3.69
		2	1.18	1.16	0.33	0.00	0.00	32.08	0.04	0.00	3.69
24	22.2	1	17.70	17.72	0.00	0.17	0.00	0.45	0.40	0.13	7.57
		2	17.55	18.01	0.01	0.00	0.00	0.00	0.39	0.12	7.69
48	45.3	1	15.00	16.40	0.00	0.00	0.00	0.00	0.30	0.28	9.09
		2	15.90	16.59	0.00	0.00	0.00	0.00	0.29	0.15	9.07
72	68.8	1	12.30	14.00	0.00	0.00	0.00	0.00	0.27	0.30	8.87
		2	12.50	14.12	0.00	0.00	0.00	0.00	0.26	0.31	7.84
96	92.4	1	12.10	11.21	0.00	0.00	0.00	0.02	0.12	0.33	10.47
		2	12.40	11.65	0.00	0.00	0.00	0.00	0.03	~~0.33~~	10.62

Strikethrough text is used when data was missing or anomalous and duplicate data was used for calculation purposes

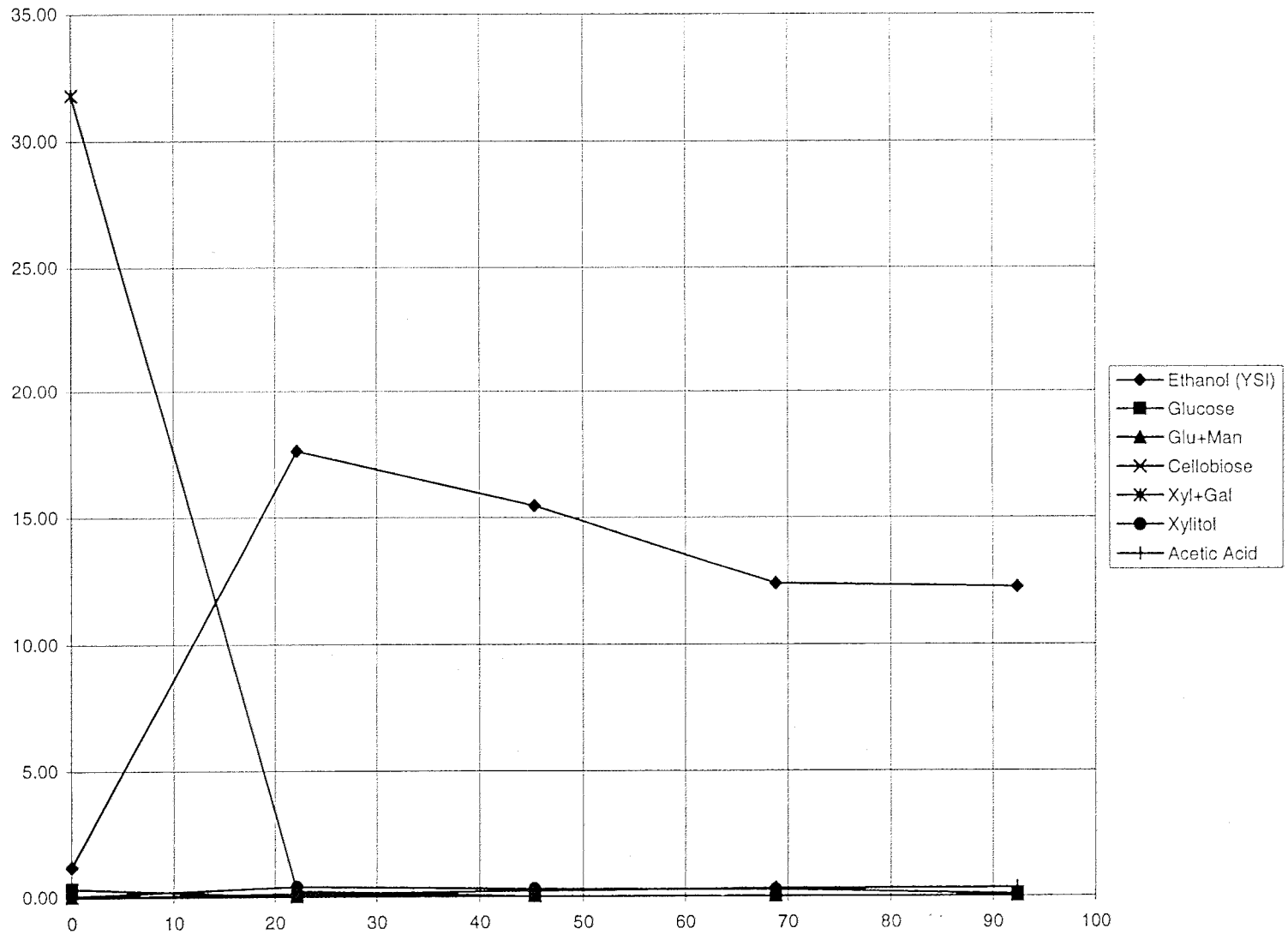

Figure 48 Products in Pichia stipitis fermentation of switchgrass reaction 3 and reaction 10, 0% hydrolysate

5.3.2 Products of Switchgrass Fermentation with Pichia stipitis

The products of *P. stipitis* fermentation of switchgrass are given in Tables 32-38 and Figures 48-54.

5.3.2.1 Switchgrass Reaction 3.

The products of switchgrass Reaction 3, 0% hydrolysate, are those given in Table 32 and Figure 48. The products of 40% hydrolysate are given in **Table 33** and **Figure 49**. Those of 80% hydrolysate are given in **Table 34** and **Figure 50**. Those of 90% hydrolysate are shown in **Table 35** and **Figure 51**.

Products in P. stipitis fermentation of switchgrass Reaction 3, 80% hydrolysate (Figure 50) shows the same increase in minor products as in the hybrid poplar Reaction 3, 80% hydrolysate. In switchgrass reaction, higher levels of cellobiose, acetic acid and xylitol are noted. Thus, the depressed rate of fermentation apparently allows more xylitol to be formed. The levels of cellobiose and acetic acid are large even in the zero time samples and may be constituents present in the hydrolysate.

The switchgrass Reaction 3, 90% hydrolysate products (Figure 51) indicates virtually no fermentation of xylose. The ethanol produced comes entirely from the glucose and mannose present. The hexoses are completely utilized by 22 hours and the ethanol concentration shows virtually no further increase beyond that time. In this reaction no xylitol is produced but relatively high levels of cellobiose and acetic acid are maintained from time zero to the end.

Table 33 Products in Pichia stipitis Fermentation of Switchgrass Reaction 3, 40% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.54	1.61	0.82	1.86	0.28	39.59	0.03	0.35	3.69
		2	1.49	1.52	0.85	1.82	0.28	39.14	0.03	0.33	3.69
24	22.2	1	20.80	21.94	0.00	1.47	0.37	0.09	0.73	0.41	9.22
		2	20.70	22.04	0.01	0.00	0.35	0.42	0.71	0.36	9.27
48	45.3	1	17.10	20.70	0.02	0.00	0.33	0.00	0.48	0.36	9.42
		2	15.20	18.27	0.01	0.00	0.32	0.00	0.44	0.32	9.75
72	68.8	1	16.20	18.03	0.02	0.71	0.47	0.42	0.78	0.41	7.77
		2	12.30	11.85	0.01	0.52	0.41	0.36	0.58	0.53	10.52
96	92.4	1	15.75	15.15	0.02	0.00	0.00	0.21	0.61	0.42	9.09
		2	~~15.15~~	14.40	0.01	0.00	0.00	0.00	1.13	0.33	13.65

Strikethrough text is used when data was missing or anomalous and duplicate data was used for calculation purposes

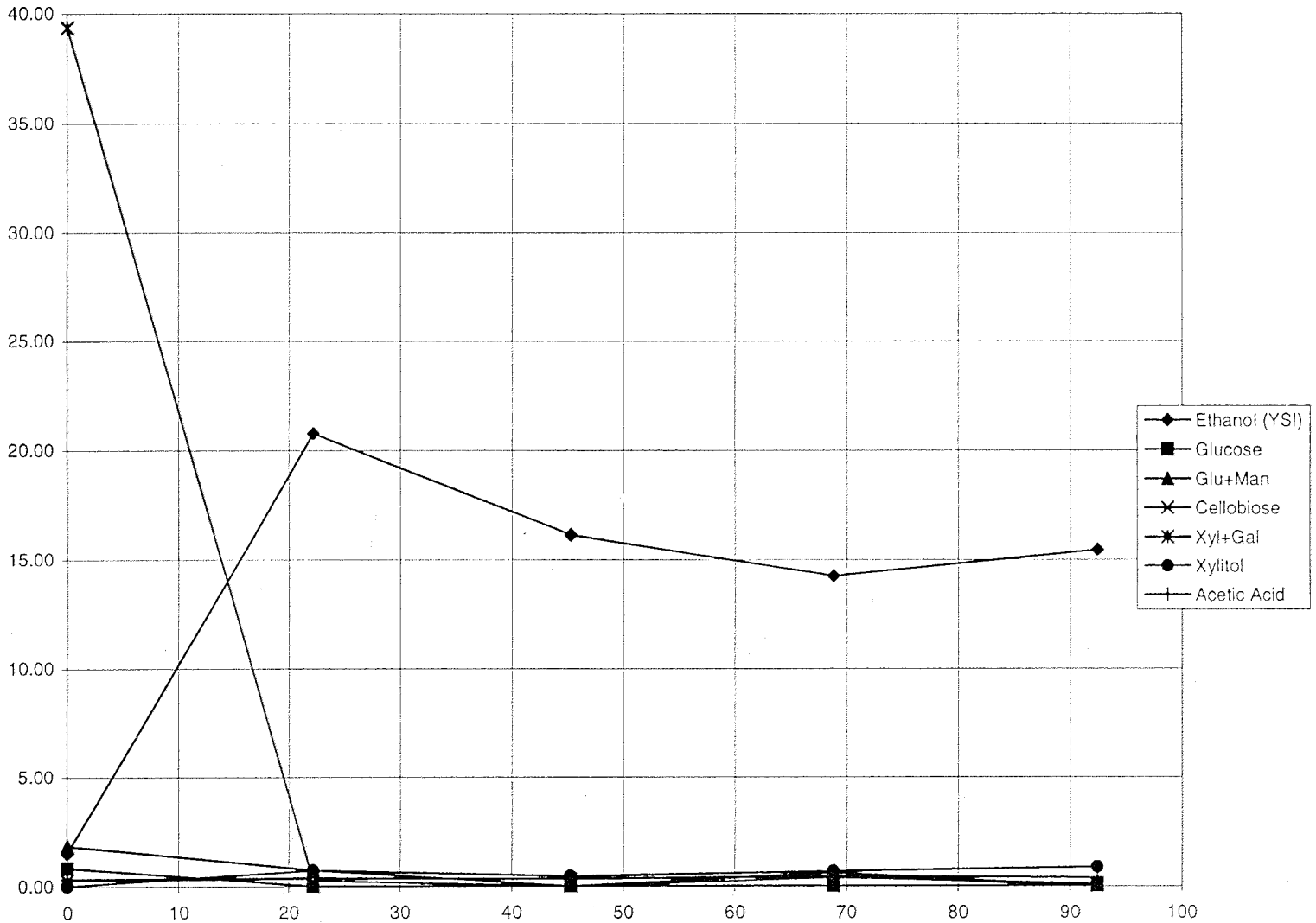

Figure 49 Products in Pichia stipitis fermentation of switchgrass reaction 3, 40% hydrolysate

Table 34 Products in Pichia stipitis Fermentation of Switchgrass Reaction 3, 80% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	0.83	0.75	5.43	9.89	3.01	31.21	0.03	1.45	3.69
		2	0.85	0.83	5.27	9.98	3.09	31.79	0.03	1.49	3.69
24	22.2	1	7.64	7.93	0.26	0.00	3.27	32.05	0.03	1.98	3.83
		2	7.60	7.88	0.24	0.00	2.73	29.46	0.03	1.44	4.01
48	45.3	1	12.00	13.03	0.25	0.00	3.27	22.64	1.71	1.80	5.34
		2	10.60	12.98	0.22	1.23	3.18	22.53	1.70	1.78	5.21
72	68.8	1	17.90	20.73	0.12	0.00	3.68	13.42	2.22	1.74	4.48
		2	16.20	18.17	0.12	1.52	3.18	11.56	1.93	1.48	4.86
96	92.4	1	18.85	19.21	0.09	0.00	2.80	3.86	1.86	1.21	5.59
		2	18.95	20.33	0.06	1.38	2.70	5.00	0.61	0.74	4.94

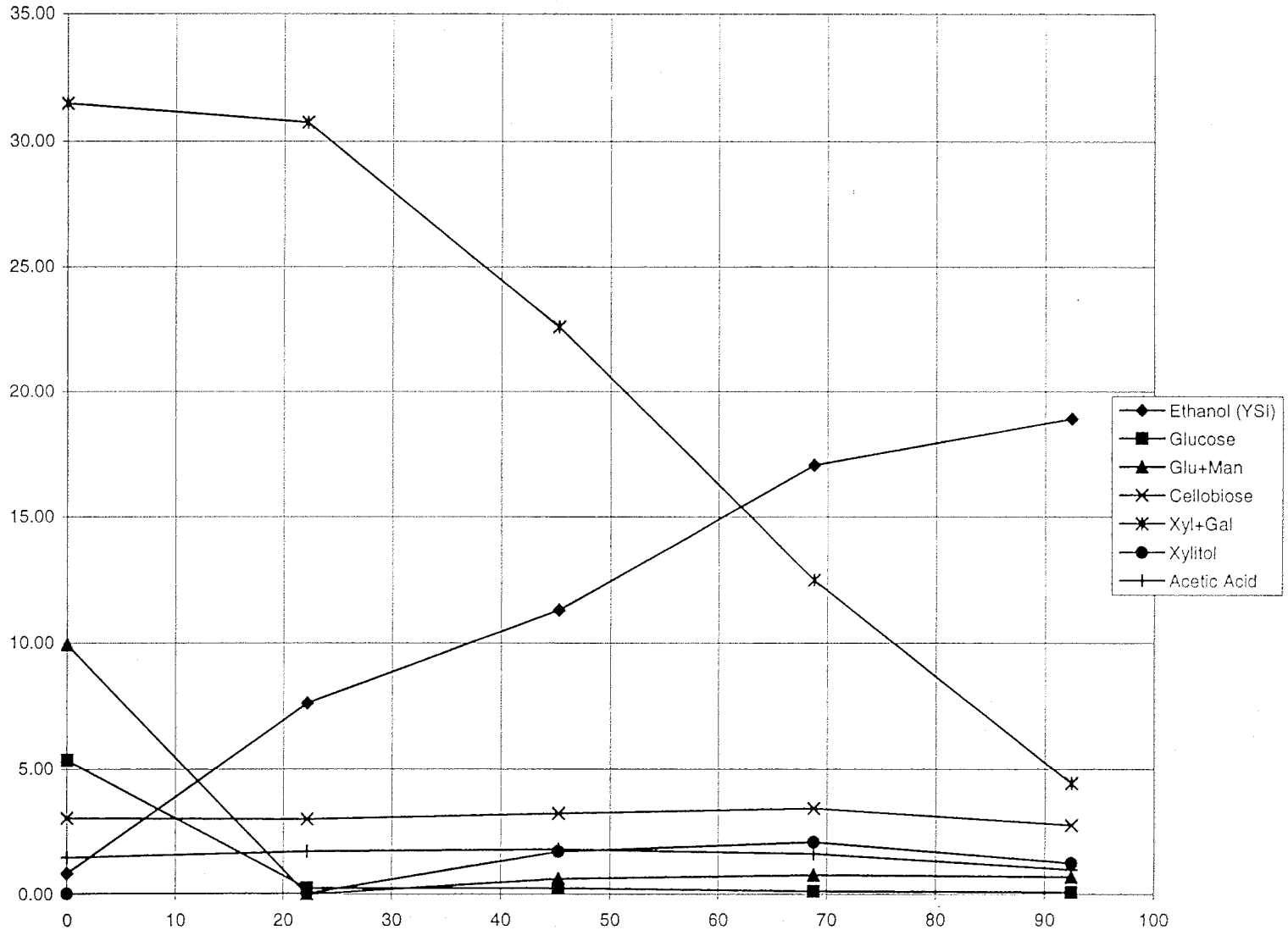

Figure 50 Products in Pichia stipitis fermentation of switchgrass reaction 3, 80% hydrolysate

Table 35 Products in Pichia stipitis Fermentation of Switchgrass Reaction 3, 90% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	0.85	0.69	5.46	11.33	3.21	28.05	0.03	1.70	3.69
		2	0.81	0.71	5.14	14.98	3.59	31.90	0.03	2.00	3.69
24	22.2	1	4.76	5.78	0.30	0.00	3.29	31.82	0.03	1.82	3.41
		2	5.44	5.81	0.01	0.54	3.35	31.80	0.03	1.79	3.36
48	45.3	1	4.90	5.22	0.37	1.02	3.57	32.68	0.03	2.34	4.33
		2	6.89	6.94	0.26	0.00	3.47	29.31	0.03	2.35	4.41
72	68.8	1	4.71	5.12	0.44	2.76	3.64	32.84	0.03	2.58	2.98
		2	6.87	7.19	0.22	0.00	3.68	28.50	0.03	2.42	3.76
96	92.4	1	4.93	5.33	0.19	0.00	3.34	31.39	0.03	2.21	3.93
		2	7.39	7.82	0.12	0.00	3.31	24.46	0.03	2.11	3.91

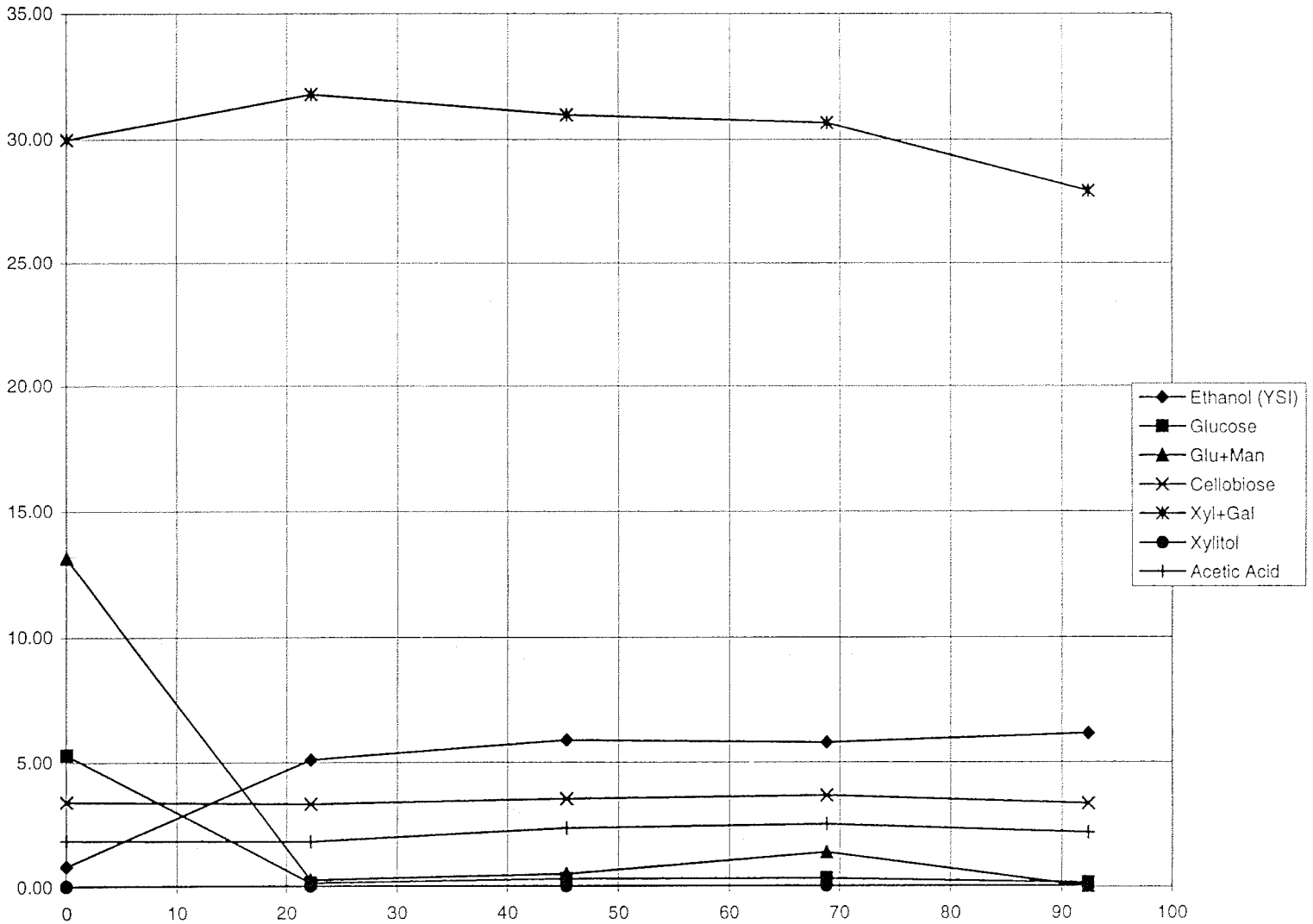

Figure 51 Products in Pichia stipitis fermentation of switchgrass reaction 3, 90% hydrolysate

5.3.2.2 Switchgrass Reaction 10

The products of switchgrass Reaction 10, 0% hydrolysate, are the same as those given in Table 32 and Figure 48. The products of 40% hydrolysate are given in **Table 36** and **Figure 52**. Those of 80% hydrolysate are given in **Table 37** and **Figure 53**. Those of 90% hydrolysate are shown in **Table 38** and **Figure 54**.

The products from switchgrass Reaction 10 hydrolysate fermentation are similar to those from Reaction 3. The major difference is that Reaction 10, 90% hydrolysate gave significant yield of ethanol by utilizing xylose. Xylitol was also produced.

Table 36 Products in Pichia stipitis Fermentation of Switchgrass Reaction 10, 40% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.32	1.08	1.11	3.07	0.57	34.64	0.03	0.68	3.69
		2	1.34	1.05	0.00	3.03	0.58	34.62	0.03	0.71	3.36
24	22.2	1	15.70	15.92	0.07	0.00	0.00	6.22	0.89	0.68	6.39
		2	16.15	16.38	0.05	0.00	0.00	5.11	0.81	0.68	6.56
48	45.3	1	14.00	17.32	0.02	0.00	0.59	3.99	1.02	0.85	6.51
		2	15.20	18.89	0.03	0.00	0.00	0.18	1.06	0.00	6.11
72	68.8	1	17.40	16.55	0.03	~~0.05~~	0.66	1.77	1.99	0.59	4.96
		2	16.50	16.81	~~0.03~~	0.05	0.30	0.00	1.16	0.09	4.96
96	92.4	1	14.00	15.85	0.02	0.00	0.00	0.00	1.17	0.13	4.84
		2	14.05	15.89	0.02	0.00	0.00	0.00	1.17	0.13	4.96

Strikethrough text is used when data was missing or anomalous and duplicate data was used for calculation purposes

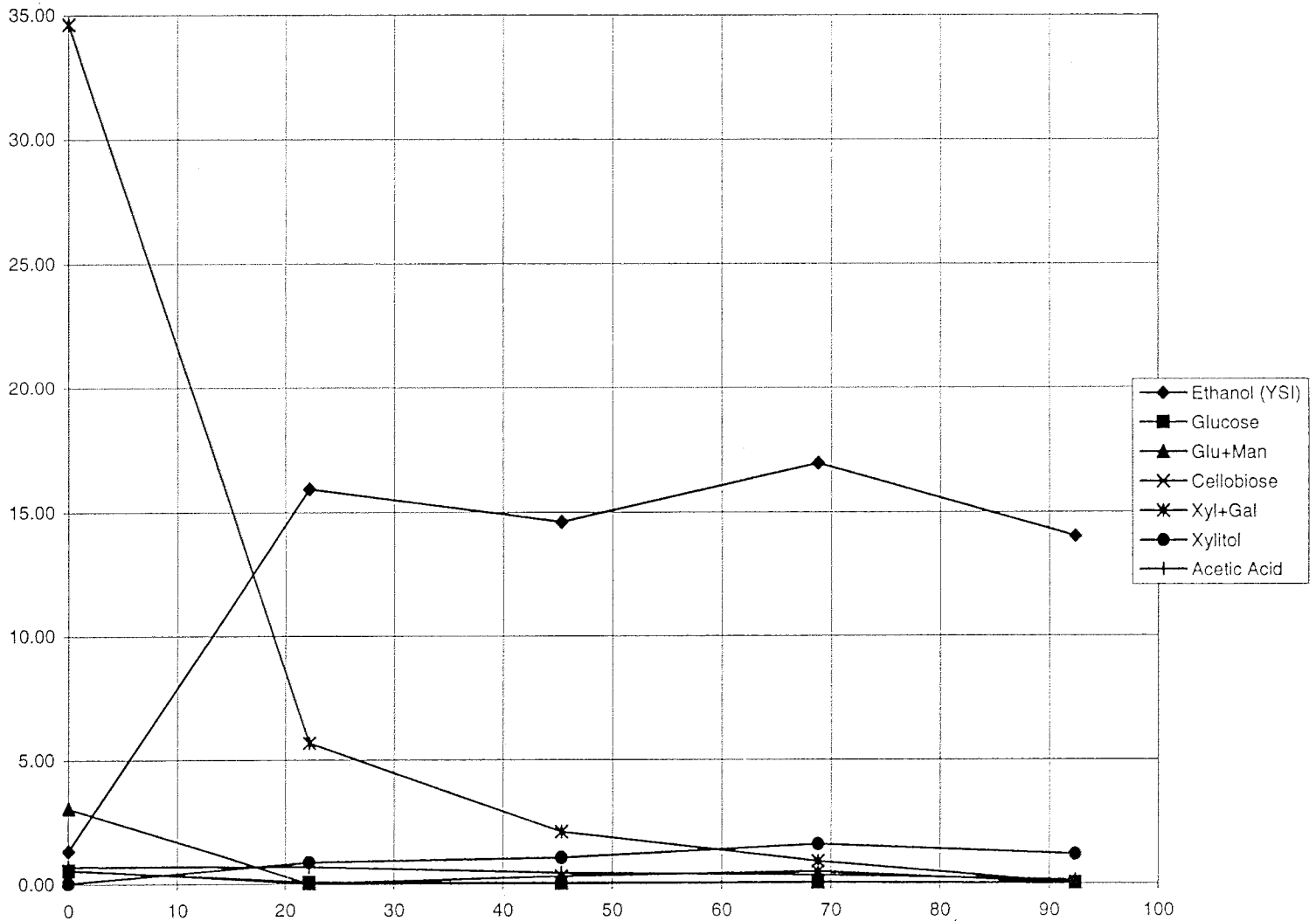

Figure 52 Products in Pichia stipitis fermentation of switchgrass Reaction 10, 40% hydrolysate

Table 37 Products in Pichia stipitis Fermentation of Switchgrass Reaction 10, 80% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.26	0.86	2.56	7.90	0.77	35.43	0.03	1.39	3.69
		2	1.20	0.88	~~2.56~~	7.81	0.81	35.36	0.03	1.37	3.69
24	22.2	1	8.74	8.80	0.20	1.00	0.40	22.03	0.59	1.55	4.56
		2	9.11	9.38	0.17	0.00	0.36	19.62	1.19	1.50	4.28
48	45.3	1	15.30	17.37	0.07	1.20	0.40	3.68	2.42	1.36	5.09
		2	13.90	17.86	0.05	0.60	0.20	2.71	2.33	1.38	5.76
72	68.8	1	15.00	16.98	0.04	1.45	0.77	0.98	3.40	2.14	4.61
		2	19.10	16.75	0.02	0.15	0.50	0.00	2.06	1.62	4.58
96	92.4	1	15.20	15.15	0.02	0.00	0.43	0.66	2.16	1.45	4.76
		2	14.60	15.83	0.02	1.09	0.72	2.60	2.57	1.67	4.89

Strikethrough text is used when data was missing or anomalous and duplicate data was used for calculation purposes

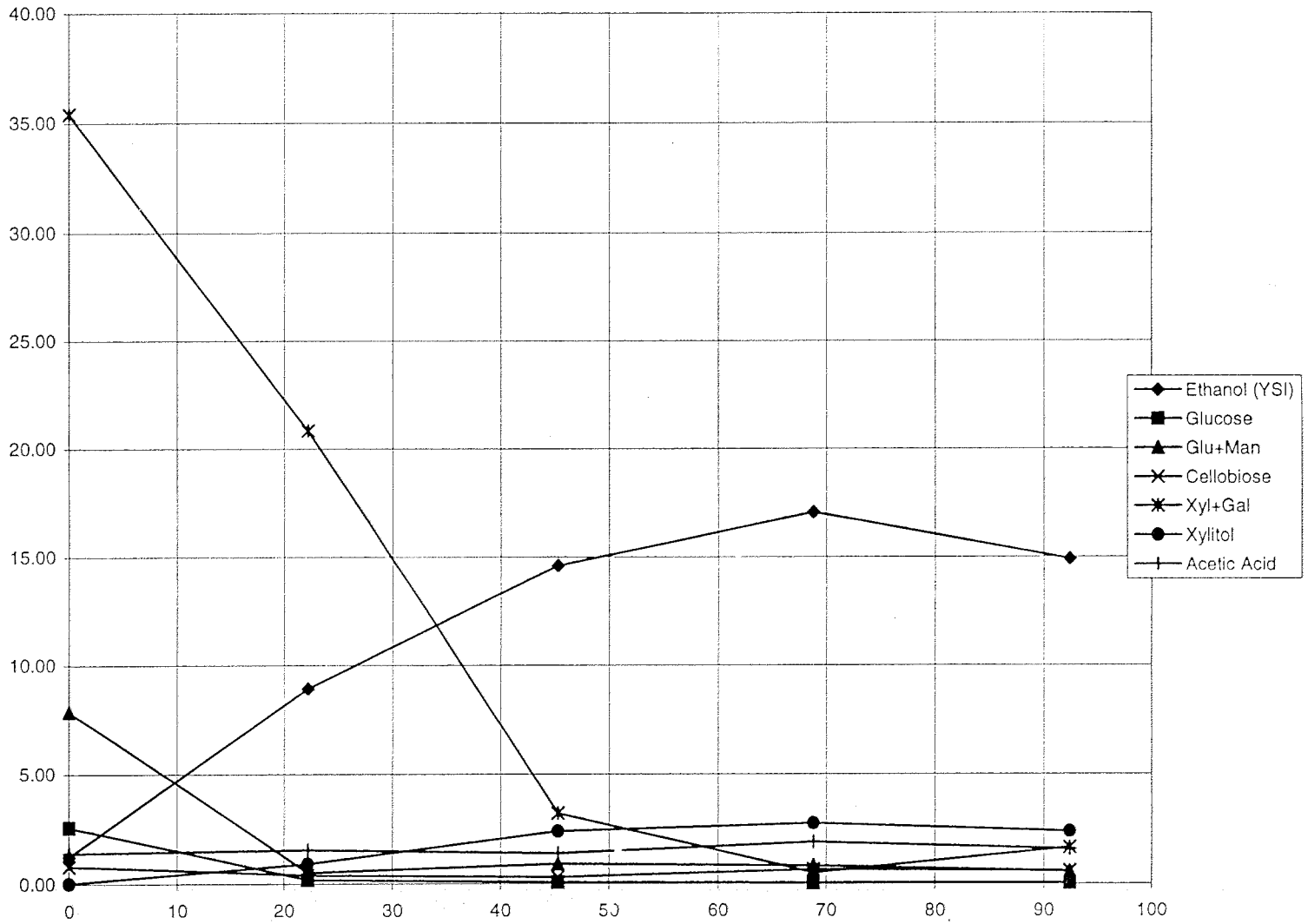

Figure 53 Products in Pichia stipitis fermentation of switchgrass Reaction 10, 80% hydrolysate

Table 38 Products in Pichia stipitis Fermentation of Switchgrass Reaction 10, 90% Hydrolysate

Concentration (g/l)

Time Nominal (hours)	Time Actual (hours)	Sample No.	Ethanol YSI	Ethanol HPLC	Glucose YSI	Glu+Man HPLC	Cellobiose HPLC	Xyl+Gal HPLC	Xylitol HPLC	Acetic Acid HPLC	DCM
0	0	1	1.19	0.85	2.74	9.32	0.08	33.19	0.03	1.52	3.69
		2	1.16	0.86	~~2.74~~	9.35	0.92	33.28	0.03	1.50	3.69
24	22.2	1	7.76	8.29	0.17	0.00	0.32	17.38	0.83	1.72	4.46
		2	8.14	8.04	0.16	0.90	0.48	18.46	0.92	1.69	4.31
48	45.3	1	13.50	14.39	0.05	0.69	0.39	4.28	2.10	1.71	5.01
		2	13.70	14.71	0.04	1.12	0.45	4.04	2.26	1.68	4.81
72	68.8	1	14.40	14.40	0.02	1.86	0.82	1.99	3.15	2.42	4.61
		2	13.40	14.36	0.02	1.84	0.78	2.01	3.26	2.42	4.66
96	92.4	1	12.85	14.27	0.02	1.38	0.76	1.51	0.22	1.78	5.11
		2	13.20	10.55	0.01	1.00	7.04	2.48	1.90	1.45	4.76

Strikethrough text is used when data was missing or anomalous and duplicate data was used for calculation purposes

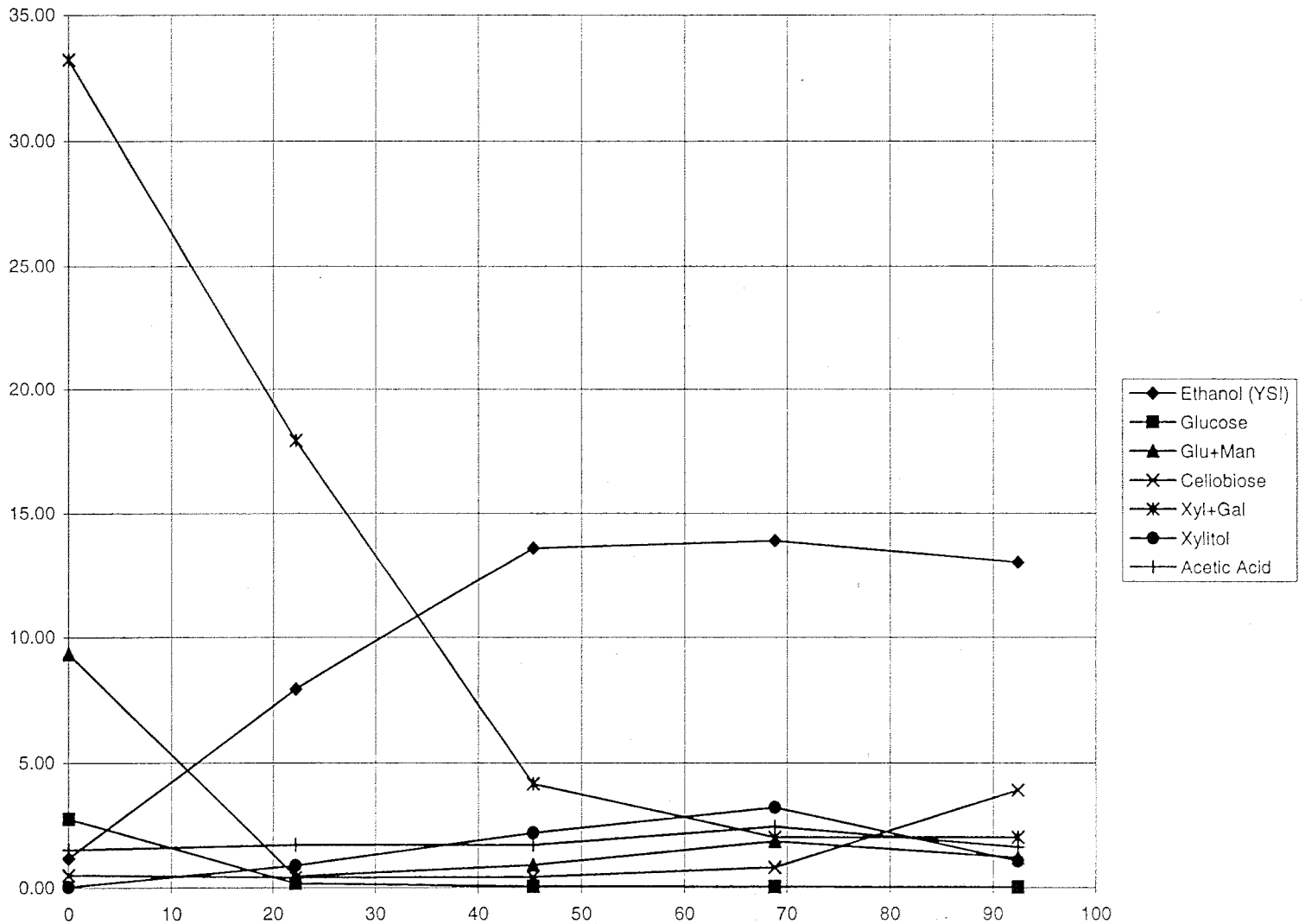

Figure 54 Products in Pichia stipitis fermentation of switchgrass Reaction 10, 90% hydrolysate

5.4 Switchgrass Extractives

Determination of % extractives of -1/8-inch (-40 Mesh) switchgrass initiated during May, 1995. This analysis was carried out using NREL's LAP-010, "Standard Method for the Determination of Extractives in Biomass", T. Ehrman 4/22/94. Aspects of the work were continued from June through September, 1995. Results of analyses made are given in **Table 39**.

It is to be noted that four samples were run for the -1/8 inch switchgrass and the -3/8 inch switchgrass feedstock supplies. The ash analyses of these two materials is discussed in Section 3.4.1 and given in Table 1.

In Section 4.4.2, in the discussion concerning the substantially higher average percentage of theoretical conversion of unextracted switchgrass feedstock to ethanol compared to hybrid poplar could be due to the presence of a fermentable component in the extractives of switchgrass. An analysis of the extractives of switchgrass was not included in the Experimental Plan and thus beyond the scope of the subcontract. Determination of the potential fermentability and/or composition of switchgrass extractives might obtain a definitive answer to this question. Since the extractives of grasses, in general, may contain significant amounts of soluble carbohydrates it is anticipated that switchgrass is no exception.

5.5 Power Requirements

The rpm at which the disintegrator was operated in Reaction 3, presented in Figure 10, shows an essentially constant value of approximately 420-rpm. This should be reflected in the power drawn by the disintegrator shown in Figure 11. The greater variability in power shown in Figure 11 may be attributed to at least two different phenomenon. Firstly, long-term random changes may occur in the consistency of the slurry entering the disintegrator. This would account for the increase in power in the interval of approximately 22:30 to 22:50 hours. Secondly, short-term cyclic changes in friction in the annular space between the bearing surface (stator) and the shaft (rotor) of the disintegrator, which provides pressure integrity in the reactor. Water is pumped to purge solid particles from the annular space. The majority of this purge water is discharged through the packing at the bottom of the disintegrator. A small portion is discharged from the annular space into the disintegrator. As the solids accumulate in the annular space the pressure of water builds up until particles discharge relieving the pressure and lowering the power required to maintain the rpm of the disintegrator shaft. (Brink, 1995, Section 4.2.2, p. 37 and Brink, D.L., M.M. Merriman, and D.A. Mixon, 1987)

The power required by the disintegrator is illustrated in Figure 11 for Reaction 3. It was shown that the power consumption was essentially constant at about 330 watts from F.S. to F.E., the time that slurry is being processed through the disintegrator.

Relating this power to that required to operate the disintegrator was not established. In work previously carried out using New York hardwoods as the feedstock it was shown that as the hydrolysis of the hemicelluloses in the feedstock proceeded the power requirements were substantially reduced. In reactions carried out in water with no acid added but at reaction temperature, production of slurry was very slow and power requirements increased substantially (Brink, 1993).

Table 39. Extractives Determination of Switchgrass Feedstock

(-1/8" Switchgrass)			
Sample	Extractive Content	Statistics Summary	
1	12.27	Mean	12.11
2	12.1	Std. Error	0.07
3	11.92	Median	12.13
4	12.15	S.D.	0.15
		Sample Var.	0.02

(-3/8" Switchgrass)			
Sample	Extractive Content	Statistics Summary	
1	13.57	Mean	13.38
2	13.05	Std. Error	0.18
3	13.1	Median	13.34
4	13.8	S.D.	0.37
		Sample Var.	0.13

6. Pretreated Substrate Samples

Samples of the best, hybrid poplar, Reaction 1 and the worst hybrid poplar pretreatment residues were submitted to NREL the summer of 1995. Submission of switchgrass samples was deleted as a deliverable by NREL.

7. Attend Annual Ethanol Project Review Meeting

Plans were completed for attending the meeting with the subcontractors and the meeting was attended May 11 and 12 in Golden, Colorado. Two presentations were made on May 12. Task completed.

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